INFECTION AND IMMUNITY, Sept. 1994, p. 3984–3993
0019-9567/94/$04.00+0

Vol. 62, No. 9

Characterization of Defined *ompR* Mutants of *Salmonella typhi*: *ompR* Is Involved in the Regulation of Vi Polysaccharide Expression

DEREK PICKARD,[1] JINGLI LI,[1] MARK ROBERTS,[1] DUNCAN MASKELL,[2] DAVID HONE,[3] MYRON LEVINE,[3] GORDON DOUGAN,[2] AND STEVE CHATFIELD[1]*

Medeva Vaccine Research Unit, Department of Biochemistry,[1] and Department of Biochemistry,[2] Imperial College of Science, Technology and Medicine, London SW7 2AY, United Kingdom, and Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201[3]

Received 12 April 1994/Returned for modification 3 May 1994/Accepted 15 June 1994

The *ompB* operon, comprising the *ompR* and *envZ* genes, was cloned from a *Salmonella typhi* Ty2 cosmid bank and characterized by DNA sequence analysis. The *S. typhi ompR* and *envZ* genes contained open reading frames encoding proteins of 240 and 451 amino acids, respectively. Comparison with the *Salmonella typhimurium* OmpB protein sequences revealed 99.5% homology. The DNA sequence data were used to identify appropriate restriction sites for generating a defined deletion of 517 bp within the open reading frame of the *ompR* gene. This deletion was introduced by homologous recombination into the chromosomes of two *S. typhi* strains which already harbored defined deletions in both the *aroC* and *aroD* genes. The presence of the deletions within *ompR* was confirmed by Southern hybridization and sequencing of the DNA fragments surrounding the deleted regions by PCR. The *S. typhi ompR* mutants displayed a marked decrease in OmpC and OmpF porin expression as demonstrated by examination of outer membrane preparations. It was also found that *S. typhi* strains harboring the defined *ompR* deletions no longer agglutinated with Vi antiserum. However, when a functional *ompB* operon was introduced back into the *S. typhi ompR* mutants, either on a multicopy plasmid or as a single-copy chromosomal replacement, the Vi^+ phenotype was restored. The levels of Vi synthesis were also found to be sensitive to different concentrations of sodium chloride present in the growth medium, although the levels of sensitivity varied between different isolates of *S. typhi*. It is therefore concluded that the *ompR-envZ* two component regulatory system plays an important role in the regulation of Vi polysaccharide synthesis in *S. typhi* and that one of the environmental signals for this regulation may be osmolarity.

Advances in molecular genetics have facilitated an increasing understanding of how microbial pathogens survive in the host and cause disease. Progress has stemmed from the identification of numerous genes from microbial pathogens whose products are essential for host colonization and survival, coupled with the ability to manipulate these genes to introduce defined deletions back into pathogenic strains. The phenotypic effects of a mutation on virulence can be determined in relevant animal or cell culture models. The knowledge gained is being utilized in the search for new and more effective prophylactic and therapeutic strategies for the control of infectious disease. One area under intensive investigation is the development of a potentially new generation of live attenuated, genetically defined vaccines. Effort has centered on the development of effective oral vaccines for enteric pathogens such as *Salmonella* spp. (9, 11, 35), *Vibrio cholerae* (36, 38), and *Shigella* spp. (40, 57). Several classes of genes that can be mutated to construct attenuated derivatives of these bacterial pathogens have been identified. These include genes for classical virulence determinants such as toxins (36, 45) or invasins (22), housekeeping genes encoding enzymes whose products cannot be assimilated from host tissues (19), and, more recently, regulatory genes that coordinately control the expression of groups of genes that are required in the host in response to environmental stimuli (2, 3, 10, 18, 44).

* Corresponding author. Mailing address: Medeva Vaccine Research Unit, Department of Biochemistry, Imperial College of Science, Technology and Medicine, Exhibition Rd., London SW7 2AY, United Kingdom.

Attenuation of virulent *Salmonella* strains can be demonstrated by evaluating *Salmonella typhimurium* mutants in the murine typhoid model (12, 19, 50). Several classes and combinations of mutants of *S. typhimurium* are attenuated in this model, including those with mutations in genes encoding key enzymes in the aromatic biosynthetic pathway (*aro* mutants [19]), those with mutations involved in a stress response (*htrA* mutants [12, 34]), and those with mutations controlling the expression of genes in response to environmental stimuli (*ompR* mutants [10]). Results from such studies have been useful in guiding the construction of suitable genetically defined attenuated *Salmonella typhi* strains that are now under evaluation as oral typhoid vaccine strains in humans (62). They have also been useful in gaining an understanding of the sophisticated gene regulatory systems employed by such pathogens during the infection process.

S. typhi causes a severe systemic illness which is communicable via ingestion of contaminated water or food containing the bacterium. It is an invasive bacterium capable of entering several types of host cells, including epithelial cells of the ileal mucosa, macrophages, and other cells and tissues of the reticuloendothelial system. *S. typhi*, like other enteric pathogens, has to respond quickly to these changing host microenvironments encountered in vivo, which will exert different demands and stresses (i.e., osmolarity, pH, oxygen tension, and nutrient starvation) on the bacterial cell. Bacteria possess systems for sensing these external environments, responding by coordinately controlling the expression of genes whose products are employed to assist survival under different conditions (17, 25, 49). The two-component regulatory systems are of

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Reference and/or source
E. coli		
HB101	F⁻ *pro leu thi hsdS*$_B$20 *endA recA rpsL20* (Str^r) *supE44 mcrB*$_B$	55
HU835	r⁻ m⁺ *c*I857 *S7 b2 red*B3	R Hull via W. J. Dallas
JM109	Δ(*lac-proAB*) *supE44* (F′ *proAB*⁺ *lacI*^{q}ZΔMI5)	J. Messing
SY327	*pir* lysogen	47
S. typhi		
CVD906	*aroC aroD* mutant of ISP1820	D. Hone (29)
CVD908	*aroC aroD* mutant of Ty2	D. Hone (29)
BRD985	*ompR* mutant of CVD908	This study
BRD989	*ompR* mutant of CVD908	This study
BRD990	*ompR* mutant of CVD908	This study
BRD1066	BRD985 harboring pTYOR2	This study
BRD1067	BRD985 harboring pTMOR1	This study
BRD1068	BRD985 with intact *ompR* gene from pTYOR5 recombined into the chromosome	This study
BRD1069	As for BRD1068	This study
BRD1071	BRD985 with deleted *ompR* gene after electroporation with pTYOR5	This study
S. typhimurium SL1344	*his*	B. Stocker (28)
Plasmids		
pTMOR1	*ompR-envZ Hgi*AI clone of SL1344 in SKII+	This study
pTYOR1	*ompR-envZ* cosmid clone from *S. typhi* Ty2	This study
pTYOR2	*ompR-envZ Hgi*AI clone of *S. typhi* in SKII+	This study
pTYOR3	Deleted *ompR* of *S. typhi* in SKII+ vector	This study
pTYOR4	*Sma*I fragment from pTYOR3 in pCVD442 vector	This study
pTYOR5	*Sma*I fragment from pTYOR2 in pCVD442	This study
pTYOR6	PCR fragment consisting of *ompR* only, cloned into pGEM-T vector	This study
pCVD442	λ*pir sacB bla*	Donneberg et al. (16)
SKII+	*bla lacZ*	Stratagene
pHC79	ColE1 *cos*⁺ Ap^r Tc^r	BRL GIBCO
pGEM-T	*bla lacZ*	Promega

importance in this sensory response (1, 51). Members of this family of regulatory genes comprise a histidine kinase sensor protein gene and a transcriptional activator gene (61). One such regulatory system, the *ompR-envZ* regulon, has recently been identified as being of importance for *Salmonella* virulence (10, 18).

Part of the adaptive response of bacterial pathogens such as *S. typhimurium* to conditions of high osmolarity is to preferentially express one type of porin, OmpC, over another type, OmpF. It has been demonstrated that mutations in *ompR* and in the porin genes that it regulates (*ompC* and *ompF*) can attenuate virulent *S. typhimurium* (18). Strains harboring such mutations were also capable of vaccinating mice against salmonellosis (10). However, it was not possible to fully mimic the in vivo properties of an *ompR* mutant by introducing mutations into *ompC* and *ompF* within the same strain, indicating that other genes under the control of the *ompR* regulatory system may contribute to *Salmonella* virulence (10).

The *ompR* regulon is also involved in the expression of virulence of other organisms. *Shigella flexneri ompR* mutants are severely impaired in their ability to invade epithelial cells, an effect thought to be mediated by the regulation of OmpC synthesis (4). Interestingly, OmpC is regulated differently in *S. flexneri*, in which OmpC is expressed constitutively under conditions of high and low osmolarity, than in *Escherichia coli*, in which it is preferentially expressed under conditions of high osmolarity (5). This has also been found to be the case for *S. typhi* (52).

In view of previous findings with *S. typhimurium* and the knowledge that the *ompR-envZ* two-component system may operate in a different manner in *S. typhi*, we investigated the effect of introducing a defined *ompR* mutation into an *S. typhi* Ty2 derivative. We report here that the *ompR-envZ* two-component system is involved in the regulation of the biosynthesis of Vi polysaccharide, a capsular antigen thought to be involved in the virulence of *S. typhi*, and that one of the environmental signals for this control may be osmolarity.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and growth conditions. The bacterial strains, plasmids, and bacteriophage used in the course of this study are described in Table 1. *E. coli* and *S. typhimurium* strains were routinely cultured on L agar or in L broth (55). *S. typhi* strains were cultured in brain heart infusion broth (Oxoid, Basingstoke, United Kingdom) supplemented with aromatic compounds as previously described (29). Broth was solidified with 1.6% Noble agar (Difco, Detroit, Mich.) for culture on agar plates. Ampicillin was used at a final concentration of 50 μg/ml when appropriate. All chemicals and antibiotics were obtained from Sigma (Poole, Dorset, United Kingdom) unless otherwise stated. Strains CVD908 and CVD906 have been described previously (29). For testing the influence of salt concentration on Vi expression, L agar and broth were prepared as usual except that the NaCl concentration was altered to the levels described in Results. All solutions used in these preparations were adjusted to pH 7.0 and were autoclaved prior to use.

Purification of DNA and manipulation techniques. Unless otherwise stated, DNA manipulations, including cosmid cloning and Southern hybridizations, were carried out as described by Sambrook et al. (55). DNA probes were labelled with [α-^{32}P]dATP (Amersham) by using the random prime labelling kit (Boehringer Mannheim, Lewes, United Kingdom). Restriction enzymes, plasmid vectors, and buffers were purchased from Boehringer Mannheim or New England Biolabs (Herts,

United Kingdom). T4 DNA ligase was purchased from BRL GIBCO (Paisley, Scotland). Chromosomal DNA of *S. typhi* was isolated by the method of Hull et al. (32), except that the crude DNA extract was incubated overnight at 50°C in the presence of proteinase K and sodium *N*-lauroyl sarcosinate. Plasmid DNA was purified by the alkaline lysis method (8). DNA fragments were purified from agarose gels by the method of Tautz and Renz (65).

Preparation of an *S. typhi* cosmid bank. An *S. typhi* cosmid bank was prepared by established methods in the cosmid vector pHC79 (55). Ligated preparations were used to transform *E. coli* HU835 by using a lambda bacteriophage packaging kit (Amersham) under the conditions recommended by the manufacturer. Seven hundred ampicillin resistant transductants were selected and grown in 75-μl aliquots of L broth, containing ampicillin, in microtiter plates at 30°C overnight. Dimethyl sulfoxide was added to a final concentration of 10% (vol/vol) to each well, and the plates were then stored at −70°C until required.

DNA sequencing. For double-stranded sequencing, DNA was isolated by the method of Stephen et al. (59). The sequencing method used was that of Sanger et al. (56), with the Sequenase 2 kit supplied by United States Biochemical Corp. (Cleveland, Ohio). DNA was labelled with α-^{35}S-dATP at 1,000 μCi/mmol, and fragments were separated by using wedge gels as previously described (7).

Electroporation of *S. typhi*. Electroporation of *S. typhi* was carried out as described previously (11). For electroporation involving suicide plasmids, colonies were initially isolated on L agar containing added aromatic compounds and ampicillin. In order to select for the second recombination event involving loss of the integrated plasmid DNA, the method of Donenberg and Kaper (16) was used. Briefly, single colonies were inoculated into L broth containing aromatic compounds and grown in a shaking incubator overnight at 37°C. The following day they were subcultured and grown to late log phase in the absence of antibiotic selection. Serial dilutions of the resulting cultures were inoculated onto L agar plates containing 5% (wt/vol) sucrose and incubated overnight at 37°C. Sucrose-resistant colonies were tested for ampicillin sensitivity, and those colonies that were sucrose resistant and ampicillin sensitive were checked for the presence of the *ompR* deletion by Southern hybridization and PCR.

DNA amplification by PCR. PCRs on *S. typhi* colonies were carried out by using *Taq* DNA polymerase and the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, Conn.) as described previously (11). The oligonucleotides used in the PCRs were derived from the 5′ end of the *ompR* sequence and a sequence complementary to the 3′ end of the *ompR* gene. These oligonucleotides, MGR06 (5′ AGTACAGACAATGCAAGA GAA 3′) and MGR07 (5′ CGAACAGCAAGGTGACGAT GA 3′), mapped at bp 272 to 292 and bp 1070 to 1050 of the *S. typhi* sequence, respectively.

A PCR clone coding for the entire OmpR protein and only 28 amino acids of EnvZ was constructed by using oligonucleotides MGR109 (5′ AGG GGC GTT TTC ATC TCG 3′) and MGR120 (5′ ACC AGG CTG ACG AAC AG 3′). These map at bp 42 to 59 and bp 1080 to 1064 of the *S. typhi* sequence, respectively. The PCR fragment was cloned into vector pGEM-T (Promega, Southampton, United Kingdom). The resulting plasmid was designated pTYOR6. This isolate also contained the promoter regions of *ompR*.

Immunological procedures. The presence of either Vi or 09 antigen on the *S. typhi* derivatives was assayed by slide agglutination with specific antisera obtained from Murex (Dartford, United Kingdom). For detection of 09 antigen, *S. typhi* cells were boiled for 20 min and cooled prior to addition of 09 antiserum. To confirm the absence or presence of an intracellular accumulation of Vi polysaccharide, double immunodiffusion assays were employed. These were carried out with anti-Vi antiserum in 1.0% (wt/vol) agarose in phosphate-buffered saline (PBS) containing polyethylene glycol 6000 at a final concentration of 2% (wt/vol). Bacteria were grown on L agar plates containing aromatic compounds overnight at 37°C. Cells were harvested, suspended in PBS, adjusted to an optical density at 600 nm of 20 or 60, and disrupted by sonication (Heat Systems Inc., Farmingdale, Conn.) in 30-s bursts for a total of 2.5 min. This procedure was carried out at 4°C, with 30 s between each burst to allow samples to cool.

Outer membrane protein isolation. Total cell envelopes were first obtained by sonication of aerobically grown L agar cultures of *S. typhi*, with the L agar either supplemented with NaCl at 0.3 M or not supplemented. The sonication was carried out with 5-ml aliquots of cells (optical density at 650 nm of 15) suspended in 10 mM sodium phosphate buffer (pH 7.2). The resulting sonicated material was subjected to low-speed centrifugation to remove cell debris and then spun at 100,000 × *g* for 45 min in order to pellet the cell envelopes. Finally, the inner membrane was solubilized by 1% sodium lauroyl sarcosinate treatment, and the outer membrane-enriched fraction was collected by a final centrifugation of 100,000 × *g* for 1.5 h (20). The pellets were resuspended in phosphate buffer and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% (wt/vol) polyacrylamide gels.

Nucleotide sequence accession number. The DNA sequence of *S. typhi ompR-envZ* has been submitted to the EMBL Data Library under accession number X78270.

RESULTS

Cloning and mapping of the *S. typhi ompR* and *envZ* genes. Published data indicated that the *ompR* and *envZ* genes of *S. typhimurium* were located on a 3.23-kb *Hgi*AI fragment (39). Chromosomal DNA of *S. typhimurium* SL1344 was therefore digested with *Hgi*AI and separated by agarose gel electrophoresis. The fragments in the 3.2-kb region were purified from the gel, and the ends were blunted by treatment with Klenow fragment and ligated into the *Eco*RV site of plasmid Bluescript IISK+ (pSKII+). Four hundred transformants of HB101 were then probed with a 501-bp PCR product derived from the *S. typhimurium ompR* gene and mapping at positions 392 to 893 of the published sequence (39). Bacterial colonies that hybridized with this probe were used to prepare plasmid DNA. Restriction digest patterns and further hybridization studies were performed, and one plasmid which was shown to contain the *ompR* and *envZ* genes, pTMOR1, was used for further studies. pTMOR1 was digested with *Pst*I and *Sal*I, and the 3.23-kb DNA fragment used for hybridization with *S. typhi* and *S. typhimurium* chromosomal DNA was digested with *Hgi*AI, *Sac*I-*Nar*I, and *Hgi*AI-*Bgl*II. The resulting hybridization patterns demonstrated that the *ompR* and *envZ* genes of *S. typhimurium* and *S. typhi* were located on similar-sized fragments for all the restriction enzymes used (data not shown). This indicated that there was enough similarity between the *S. typhi* and *S. typhimurium* genes to be able to employ the *Pst*I-*Sal*I 3.23-kb fragment from pTMOR1 as a probe for identifying the *S. typhi ompB* operon in the *S. typhi* cosmid bank. One colony which hybridized with this fragment harbored a cosmid with a 27-kb insert of *S. typhi* DNA. Digestion of this cosmid, pTYOR1 (Fig. 1), with *Hgi*AI yielded several fragments, including one with the expected size of 3.23 kb. This

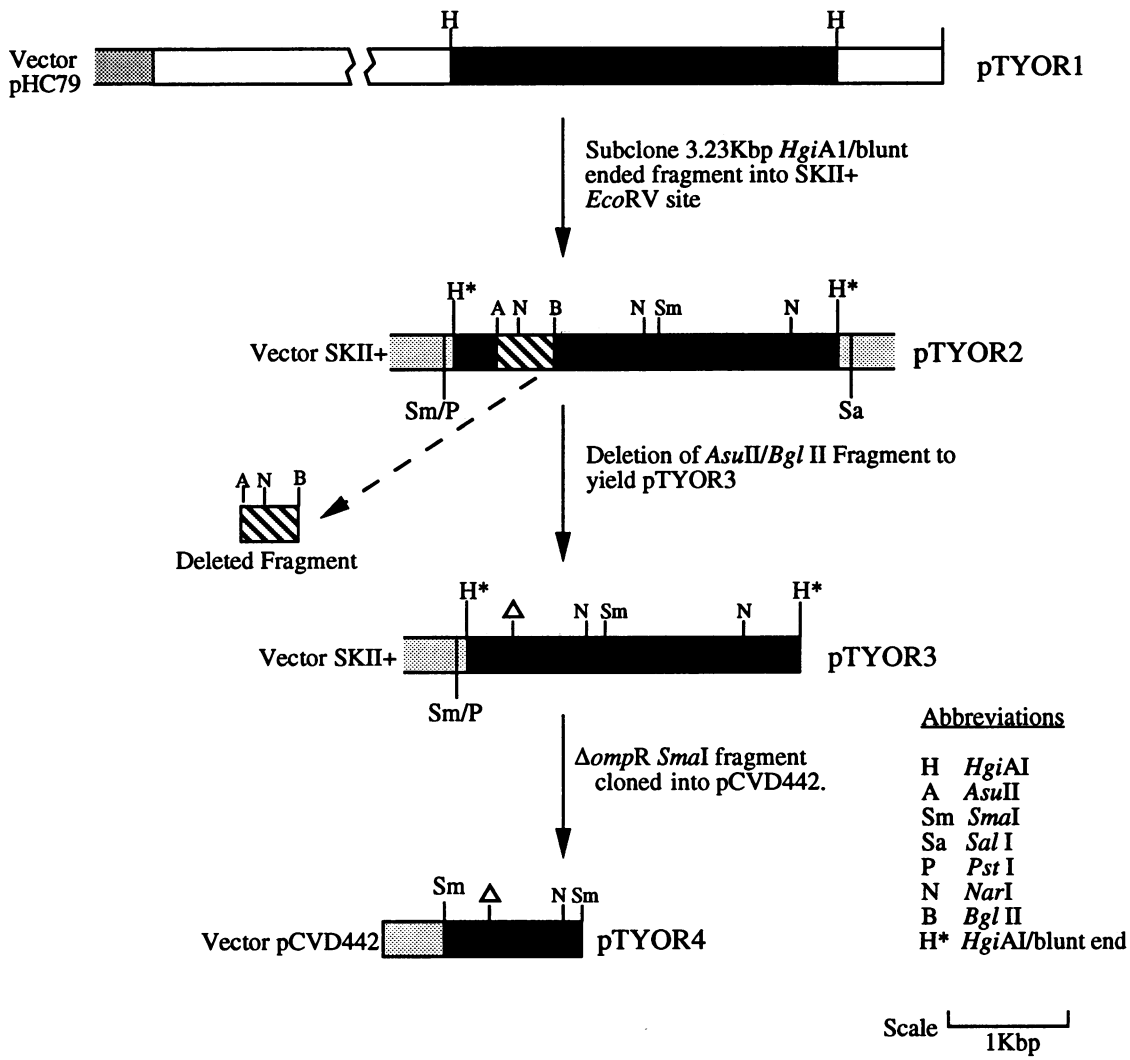

FIG. 1. Schematic diagram showing the construction of the plasmids used to make the defined deletion in the *ompR* gene. Full details are given in the text. The black shaded areas indicate *S. typhi ompB* DNA. The unshaded areas indicate *S. typhi* DNA outside the *ompB* region.

fragment was isolated and ligated into the *Eco*RV site of pSKII+ as described above. The ligation mix was used to transform HB101, and plasmid DNA was isolated. One plasmid with the expected structure was characterized by restriction mapping and Southern hybridization and confirmed to harbor the *S. typhi ompR* and *envZ* genes. This plasmid, pTYOR2, was used for further studies (Fig. 1).

Nucleotide sequence of *S. typhi ompR-envZ*. The full sequences for the *ompR* and *envZ* genes of *S. typhi* were determined and compared with those of the equivalent *S. typhimurium* genes. Analysis of the sequence revealed the presence of two open reading frames encoding 240 and 451 amino acids for *ompR* and *envZ*, respectively. The amino acid sequence encoded by the *S. typhi ompR* gene was identical to that for the *S. typhimurium* gene, and there were only two amino acid changes for the *envZ* gene.

In total, there were only 25 base pair changes of 3,237 bp in the *ompR* and *envZ* genes between *S. typhi* and *S. typhimurium*. Two base pair changes were in *ompR*, 11 were in *envZ*, and 12 were in DNA outside the *envZ* gene itself (between bp 2350 and 3237).

Construction of a defined deletion into the *S. typhi ompR* gene. DNA sequencing of the *S. typhi ompR* gene revealed the presence of unique *Asu*II and *Bgl*II sites (mapping at 375 and 893 bp, respectively) within the coding sequence which could be used to generate a deletion. pTYOR2 was therefore digested with *Asu*II and *Bgl*II, religated, and used to transform *E. coli* HB101. Plasmid DNA was purified from transformants and analyzed by restriction digestion with *Sal*I-*Pst*I, *Sma*I, and *Nar*I (Fig. 1). One plasmid, PTYOR3, was found to have the expected 517-bp deletion within the *ompR* gene and was used for further study.

Introduction of the *ompR* deletion into the chromosome of *S. typhi*. The suicide replicon pCVD442 (16) was used to introduce the *ompR* deletion into the chromosome of *S. typhi*. pCVD442 is a derivative of pGP704 which cannot replicate in *S. typhi* strains because they lack the *pir* gene (47). pCVD442 additionally harbors the *sacB* gene of *Bacillus subtilis*, which encodes the enzyme levan sucrase, the presence of which is toxic for gram-negative organisms when they are growing in the presence of sucrose. This greatly facilitates the introduction of defined deletion mutations back into the chromosome

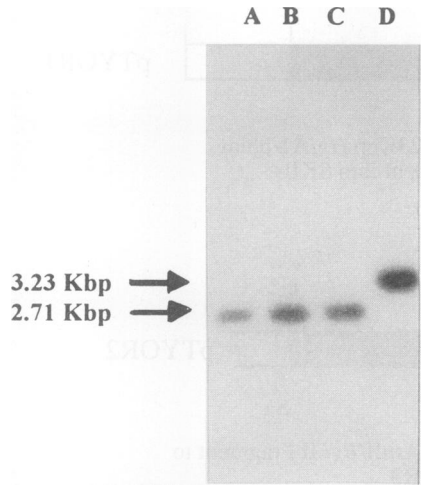

FIG. 2. Southern hybridization demonstrating the presence of the deletion in the *ompR* gene of *S. typhi*. Chromosomal DNA was cleaved with *Hgi*AI and reacted with the ^{32}P-labelled *ompR* probe. Lanes A, B, and C, *ompR* mutants BRD985, BRD989, and BRD990, respectively. Lane D, CVD908, which harbors the wild-type *ompR* gene. Molecular sizes are shown on the left.

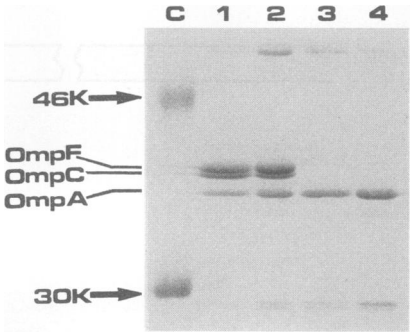

FIG. 3. SDS-PAGE analysis of outer membranes prepared from *S. typhi* strains CVD908 (lanes 1 and 2) and BRD985 (lanes 3 and 4) grown on agar without NaCl (lanes 1 and 3) or in the presence of 0.3 M NaCl (lanes 2 and 4). Lane C, molecular weight standards of ovalbumin (46,000 [46K]) and carbonic anhydrase (30K) from Amersham. The positions of the proteins corresponding to OmpA, OmpC, and OmpF are indicated on the left.

of a gram-negative organism. Once a single homologous recombination event has been achieved, it is possible to directly select for the second recombination event by growing the strains in the presence of sucrose. Only those colonies that have lost the vector sequences will be able to grow on this medium, obviating the need to screen large numbers of colonies for antibiotic sensitivity. The plasmid pTYOR3, which carries the deleted *ompR* gene, was digested with *Sma*I, and the resulting 1.13-kb fragment was gel purified and ligated into pCVD442 which had also been digested with *Sma*I. The ligation mix was used to transform *E. coli* SY327 *pir*, and a plasmid with the expected structure was identified by restriction mapping (Fig. 1) and designated pTYOR4. This plasmid was used to transform *S. typhi* CVD908 by electroporation, with transformants being selected on brain heart infusion agar containing ampicillin. Several transformants that grew stably on medium containing ampicillin were selected. These colonies were isolated and grown in L broth supplemented with aromatic compounds but lacking ampicillin. Serial dilutions of the cultures were then spread onto L agar plates supplemented with 5% (wt/vol) sucrose. Several thousand colonies that were capable of growing on this medium were isolated. Since it was not a simple task to screen directly for the *ompR* deletion by using phenotypic characteristics, we employed a method using PCR as described previously (11). We used PCR primers (MGR06 and MGR07) corresponding to regions of *ompR* outside the deleted region to directly amplify chromosomal DNA from 100 colonies. The PCR products were analyzed by gel electrophoresis. The majority of the colonies gave rise to a 799-bp product, indicating the presence of the wild-type gene. However, three colonies gave rise to a smaller 282-bp PCR product, indicating the presence of the deletion in the chromosome (data not shown). These strains were named BRD985, BRD989, and BRD990. The presence of the *ompR* deletion in these strains was confirmed by Southern hybridization (Fig. 2). An *ompR*-specific probe was prepared by labelling the *Pst*I-*Sal*I fragment of pTMOR1 and hybridizing with *Hgi*AI chromosomal DNAs of BRD985, -989, and -990 and CVD908. It can be seen that the 3.23-kb fragment for CVD908 is replaced by a smaller 2.71-kb fragment for BRD985, -989, and -990, indicating the presence of the 518-bp *ompR* deletion in these strains. This was further confirmed by using PCR to amplify and sequence a DNA fragment surrounding the deletion (data not shown).

Expression of the porins OmpC and OmpF is down regulated in the *ompR* mutant BRD985. Since OmpR is known to regulate porin expression, we investigated the effect of the *ompR* mutation on the expression of OmpC and OmpF by comparing outer membrane preparations obtained from BRD985 and CVD908. Figure 3 clearly demonstrates the down regulation of OmpC and OmpF proteins in the *ompR* mutant. This confirms that the OmpC and OmpF porins are regulated by OmpR, as suggested by the absence of complete sequences encoding the phosphorylation and DNA-binding domains in the mutation in BRD985 (48), and that this regulation is similar to that found for the porins in *S. flexneri*.

Phenotypic characterization of the *S. typhi ompR* mutants indicates that they no longer agglutinate with Vi antiserum. Routine phenotyping tests carried out on the *S. typhi ompR* mutants demonstrated that these strains no longer agglutinated with Vi antiserum. However, CVD908 did agglutinate with Vi antiserum, as did colonies that were screened for the presence of the *ompR* deletions but gave rise to the PCR product associated with the intact *ompR* gene. All the strains agglutinated with 09 antiserum, but unlike CVD908, the *ompR* mutants agglutinated without the cells having to be boiled, indicating that Vi polysaccharide was no longer masking the lipopolysaccharide antigen. *Salmonella* 08 antiserum was used as a negative control. None of the *S. typhi* strains agglutinated with this antiserum. These results suggested that the OmpR-EnvZ two-component system is involved in the regulation of Vi synthesis in *S. typhi*.

Complementation of the *ompR* mutation with pTYOR1 restores the ability to agglutinate with Vi antiserum. In order to determine if normal Vi biosynthesis could be restored by reintroducing functional *ompR* and *envZ* genes, BRD985 was transformed with pTYOR1, which contains the *Hgi*AI fragment of *S. typhi* encoding the *ompR* and *envZ* genes. Ten transformants were assessed in agglutination assays. All agglutinated with Vi antiserum to the same degree as CVD908 and required boiling before they would agglutinate with 09 antiserum. This indicated that the *ompR* and *envZ* genes present on a multicopy plasmid were able to complement the Vi$^-$

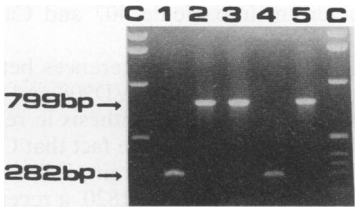

FIG. 4. Detection of isolates from the *S. typhi ompR* mutant BRD985, into which the intact *ompR* gene had been recombined back into the chromosome by homologous recombination. This was achieved by amplifying the *ompR* gene directly from whole cells by PCR. Lane 1, BRD985; lanes 2 and 3, BRD1068 and BRD1069, respectively, in which the *ompR* mutation had been replaced with the intact gene as indicated by the larger PCR product; lane 4, BRD1071, an isolate showing the Vi^- phenotype and a smaller PCR fragment; lane 5, CVD908. Molecular sizes are indicated on the left. Lanes C, 1-kbp ladder standard from BRL GIBCO.

phenotype associated with the *ompR* mutation in BRD985. Introduction of pSKII+ alone into BRD985 was not able to restore Vi biosynthesis. pTMOR1 was also able to restore Vi biosynthesis when introduced into BRD985.

Additionally, restoration of Vi synthesis was also achieved by complementing with pTYOR6, which encodes all of OmpR and only 28 amino acid residues of EnvZ. Data from DNA sequencing across the junction of the *ompR* deletion in *S. typhi* BRD985 indicated no frameshift which might have affected EnvZ activity (data not shown). This result demonstrates that it is possible to complement the mutation in BRD985 by introducing a plasmid which encodes only OmpR in the absence of a functional EnvZ.

Replacement of the *ompR* mutation in BRD985 with the intact gene by homologous recombination also restores agglutination. In order to confirm that *ompR* is involved in the regulation of Vi synthesis, the *ompR* mutation in BRD985 was replaced with the wild-type gene by homologous recombination. This was achieved by first constructing a suicide replicon harboring the complete *S. typhi ompR* gene. pTYOR2 was digested with *Sma*I and ligated into pCVD442 which had also been digested with *Sma*I. The ligation mix was used to transform *E. coli* SY327 *pir*, and plasmid DNA was purified and characterized by restriction mapping. A plasmid with the expected structure was named pTYOR5 and was used to transform BRD985. Sucrose-resistant, ampicillin-sensitive colonies were isolated as previously described. These were screened with Vi antiserum. The majority of the transformants were able to agglutinate, indicating that the *ompR* gene had replaced the deleted regions by homologous recombination. This was confirmed by employing PCR as previously described (11). Three colonies were screened in this assay, two that had regained the ability to agglutinate with Vi antiserum and one that had not. The results are shown in Fig. 4. It can be seen that the strains (BRD1068 and -1069) in which Vi synthesis has been restored gave rise to the larger PCR product (799 bp), indicating that the intact *ompR* gene had recombined into the chromosome of BRD985. An additional isolate (BRD1071) which was unable to agglutinate with Vi antiserum showed no increase in the size of its *ompR* PCR product. This also confirmed that the *ompR* and *envZ* genes are involved in the regulation of Vi synthesis.

Osmolarity regulates Vi antigen production in *S. typhi* CVD906 and CVD908. In view of the finding that *ompR* is involved in the regulation of Vi biosynthesis and that this system is known to regulate other genes, such as *ompC* and *ompF*, in response to changes in the external environment (27), we decided to investigate the effect of changes in the osmolarity of the growth medium on the synthesis of Vi polysaccharide in *S. typhi*. L agar containing a range of sodium chloride concentrations (0.0 to 0.7 M) was prepared, as previously described (64). Normal L agar contains 0.085 M NaCl. CVD908 and CVD906 were cultured on this agar, and agglutination tests were performed on organisms suspended in PBS. The findings of this study are summarized in Table 2. The main conclusions drawn from this experiment were that CVD908 no longer agglutinated with Vi antiserum when grown in medium containing 0.5 M NaCl and only weakly agglutinated at concentrations of 0.4 M NaCl. However, at NaCl concentrations below 0.4 M, all cultures agglutinated with Vi antiserum to the same degree, and 09 agglutination was not detectable without boiling of the sample first. It was therefore concluded that Vi synthesis in *S. typhi* can be regulated by external osmolarity. *S. typhi* CVD906 only weakly agglutinated with Vi antiserum, even at NaCl concentrations below 0.4 M. At 0.3 M NaCl, the CVD906 culture was only weakly Vi positive but had a strong 09 agglutination without boiling.

TABLE 2. 09 and Vi slide agglutination reactions of CVD906 and CVD908 cultures grown on L agar supplemented with different amounts of NaCl

NaCl concn (M) in L agar	Slide agglutination[a]			
	CVD906		CVD908	
	09	Vi	09	Vi
0	++	++	−	+++
0.06 to 0.17	++	++	−	+++
0.3	+++	±	+	+++
0.4	+++	−	+++	±
0.5 to 0.7	+++	−	+++	−

[a] Phenotypes were assessed by slide agglutination using Vi and 09 antisera (Murex). 09 agglutination reactions were carried out without prior boiling of cells. The degree of agglutination ranged from not detectable (−) to weak (+) to strong (+++); ± and ++ indicate intermediate degrees.

Double immunodiffusion analysis of sonicates of *S. typhi* demonstrates that *ompR* is involved in controlling the synthesis of Vi, not its surface expression. It is possible that the lack of agglutination of *ompR* mutants with Vi antiserum was due to a defect in the transport of Vi polysaccharide to the surface of the organism and that Vi was accumulating intracellularly. To investigate this possibility, we performed double immunodiffusion assays on bacterial sonicates with Vi antiserum. The results are presented in Fig. 5. CVD908 produced strong lines of identity (wells 1, 3, 5, and 7). A CVD908 culture grown on medium containing 0.5 M NaCl showed a lower level of expression of Vi antigen, as indicated by the shorter distance of the precipitation line from the antigen in well 4. The faintness of this line of precipitation is also indicative of a lower level of

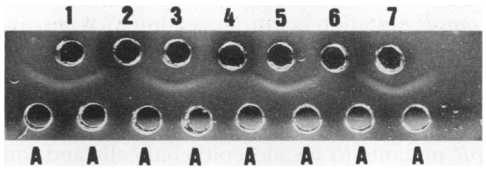

FIG. 5. Immunodiffusion patterns for sonicated *S. typhi* strains reacted with Vi antiserum. Wells 1, 3, 5, and 7, CVD908; wells 2 and 6, BRD985; well 4, CVD908 cultured in medium containing 0.5 M sodium chloride. All of the lower wells (A) contained Vi antiserum.

Vi expression. Twenty optical density units (650 nm) per ml of sonicated cells was used in both cases.

In contrast, a sonicate of BRD985 failed to produce any line of identity (Fig. 5, wells 2 and 6) even though three times as many cells were used to prepare the sonicate. This indicates that Vi polysaccharide was not being accumulated intracellularly in *ompR* mutants.

DISCUSSION

In this paper we report the finding that the *ompR* gene of *S. typhi* is involved in the regulation of the synthesis of the Vi capsular polysaccharide and that one of the environmental signals for this regulation may be osmolarity, since Vi expression is sensitive to changes in osmolarity of the growth medium.

A key theme in the coordinate regulation of different classes of bacterial genes in response to environmental stimuli is the involvement of the family of two-component systems (1, 51, 61). Included in this family are the *cheA-cheY* system in *E. coli* and *Salmonella* spp.(60), which controls chemotaxis; the *ntrB-ntrC* (42) and *phoR-phoB* (66) systems in *E. coli*, which respond to nitrogen limitation and phosphate; and the *virA-virG* system in *Agrobacterium* spp., which responds to plant exudates (58).

There are now several examples for which it has been demonstrated that interfering with the ability of bacterial pathogens to operate these sensory systems results in attenuation in vivo. *Salmonella* strains harboring mutations in the *phoP-phoQ* regulatory system have been found to be attenuated and immunogenic in the murine model (46). The *pho* regulon is responsive to phosphate levels and other conditions such as those encountered intracellularly within macrophages, an example being low pH (21). We have previously reported that mutations in *ompR* and the porin genes that it controls (*ompC* and *ompF*) also attenuate virulent *S. typhimurium* strains (10, 18). It has recently been demonstrated that the *ompR-envZ* two-component system is also involved in the virulence of *S. flexneri*. It has been found that mutants with mutations in the *ompB* operon are impaired in virulence both in vivo and in vitro, being unable to colonize epithelial cells in tissue culture (4). Further work indicated that defined *ompC* mutants behaved in a similar manner, and it was concluded that OmpC is involved in the invasion of epithelial cells by *S. flexneri* (5). This is in contrast to the findings for *S. typhimurium*, in which it was not possible to mimic the effects of the *ompR* mutation by introducing defined mutations into *ompC* and *ompF* (10). This may be related to the finding that *ompC* in *S. flexneri* is regulated differently from *ompC* in *E. coli* and *S. typhimurium*. OmpC is constitutively expressed under both high- and low-osmolarity conditions in *S. flexneri*, whereas it is expressed at increased levels under conditions of high osmolarity in *E. coli* and *S. typhimurium*. It has now been shown that *S. typhi* regulates OmpC expression in a manner similar to that of *S. flexneri* (52). The present study has also demonstrated that the OmpC and OmpF porins are both down regulated when an *ompR* mutation is introduced into *S. typhi*, and this is also similar to the results obtained with *S. flexneri* (52). In view of these findings and the recent report that osmolarity of the growth medium can affect the ability of *S. typhi* to adhere to and invade epithelial cells (64), we assessed the ability of *S. typhi ompR* mutants to invade epithelial cells and found that they were not impaired in this virulence trait (data not shown). For these studies the *S. typhi ompR* mutant and CVD908 control were grown in medium containing 0.3 M NaCl, since this level has been shown to be the optimal osmolarity for invasion of this bacterium into Henle 407 and Caco-2 cells (64).

It was interesting that there were differences between the two *S. typhi* strains used in this study (CVD908 and CVD906) with regard to their regulation of Vi synthesis in response to osmolarity. This is probably related to the fact that CVD908 is derived from the classic *S. typhi* Ty2 strain, isolated in 1916 (29), while CVD906 is derived from ISP1820, a recent *S. typhi* isolate (29). In studies in which these strains have been tested for safety in humans, it was concluded that ISP1820 may be more virulent than Ty2 (62). This may be a result of changes in Ty2 brought about by multiple passage in the laboratory and the way in which the strain has been stored since its original isolation. It is therefore not surprising that other phenotypic traits of Ty2, such as subtle changes in the regulation of Vi polysaccharide, differ from those of a modern isolate such as ISP1820.

The range of salt concentrations chosen for the osmolarity tests is thought to be relevant to both the in vitro and in vivo environments encountered by *S. typhi* during different stages of pathogenesis. Outside the host the organism is found mainly in an aqueous environment which is thought to contain no greater than 0.06 M NaCl (64). In the intestinal lumen the osmolarity is high, with values believed to be equivalent to 0.3 M NaCl and greater (64). At this osmolarity, the adherence and invasiveness of *S. typhi* into tissue culture cells have been found to be maximal (64). Once *S. typhi* has passed through the epithelial barrier into the bloodstream, it encounters an osmolarity equivalent to 150 mM NaCl (47).

In this study it was demonstrated that both *S. typhi* CVD906 and CVD908 showed a marked decline in Vi antigen synthesis at and above NaCl concentrations of 0.3 and 0.4 M, respectively, although it could still be detected at low levels at these osmolarities. This suggests that Vi may be down regulated in the gut, facilitating interactions of *S. typhi* with epithelial cells, while it is up regulated in the blood, where it is known that Vi is important for survival of the organism at this stage of the infection process.

There are now several reports describing genes encoding bacterial exopolysaccharide antigens that are regulated by two-component sensory systems. For example, in *Pseudomonas aeruginosa*, it has been found that *algR*, an environmental response regulator gene with a high degree of homology to *ompR*, regulates the transcription of *algD*, which is necessary for alginate production (13–15). *P. aeruginosa* causes severe problems in cystic fibrosis patients, who are predisposed to pulmonary infection by this organism. Organisms isolated from the lungs soon after infection do not have the mucoid phenotype associated with alginate production. However, after prolonged infection, *P. aeruginosa* shifts to the mucoid phenotype, compounding the problems for the cystic fibrosis patient (24). Moreover, it has been found that one of the signals for this phenotypic switch may be the high-osmolarity environment of the cystic fibrosis lung (6). In this study we have demonstrated that another capsular antigen from a bacterial pathogen, Vi in *S. typhi*, is also regulated by osmolarity. It is interesting that OmpR is capable of activating the *algD* promoter nearly as well as AlgR (6), which demonstrates the cross talk that can occur between different two-component systems in their responses to external stimuli (67). These interactions, in a wider context, give the bacterium greater diversity and flexibility in controlling gene expression in different host microenvironments.

In *E. coli*, the two-component system *rcsB-rcsC* regulates the synthesis of colanic acid (23); RcsC is the histidine kinase sensor phosphorylating RcsB, which acts as the transcriptional activator. As yet it is not clear which environmental signal *rcsC*

responds to. This system has also been shown to regulate the expression of K30 and K54 polysaccharide antigens in pathogenic *E. coli* (33, 54). Like Vi in *S. typhi*, these antigens are also thought to be involved in conferring resistance against complement-mediated serum killing. Interestingly, it has also been demonstrated that *rcsB* corresponds to *viaA* (31), one of the two genes known to be necessary for Vi expression in *S. typhi* and which is also present in *E. coli* and *Salmonella* strains which do not produce Vi. The other gene, *viaB*, encodes the structural genes specific for Vi expression (26) and is not expressed in *viaA* mutants of *S. typhi* or when cloned into *rcsB* mutants of *E. coli* (31). Furthermore, it has now been shown that *rcsB* cloned from *E. coli* can complement *viaA* mutations in *S. typhi* and restore Vi synthesis to these strains (31). It was concluded from these studies that *S. typhi* employs the same regulatory proteins to regulate Vi synthesis as *E. coli* uses to regulate colanic acid synthesis. The results reported here indicate that another two-component system, *ompR-envZ*, possibly responding to a different environmental signal, is also involved in the regulation of Vi synthesis in *S. typhi*. This is not surprising given the complexity of capsule regulation and the cross talk that occurs between different regulatory systems. For example, it is known that there is another regulatory component, RcsA, in addition to RcsB and RcsC, which is also involved in the regulation of colanic acid production and type 1 and type 2 capsule production in *E. coli* and *Klebsiella pneumoniae* (23). It acts in concert with RcsB as a positive regulator for colanic acid but as a negative regulator for the type 2 capsule, K54, in *E. coli*. Similarly, regulatory elements other than AlgR which also play a role in the regulation of the *algD* promoter of *P. aeruginosa* have been found (37, 68, 69). Such coregulation could be important to an invasive organism like *S. typhi*, which has to encounter and react to many microenvironments within the host. Thus, it is possible that *S. typhi* may need to regulate the synthesis of Vi antigen in the host in response to these different environments.

Vi polysaccharide is considered to be an important virulence determinant of *S. typhi*, since the majority of *S. typhi* strains isolated from the blood of patients with typhoid possess this antigen (53) and since Vi-positive strains are more virulent than Vi-negative strains both in mice and in human volunteers (30). It has also been demonstrated in vitro that Vi antigen is essential for the survival of *S. typhi* in human serum (41), possibly because of Vi reducing the rate of complement activation by the alternative pathway, thereby reducing complement-mediated killing and opsonization (41). This may be linked to the fact that Vi antigen has been found to decrease the level of fixation of the C3b component of complement (41). The in vivo relevance of these findings to the mechanisms of host defense and pathogenesis of *S. typhi* still remain speculative, and nothing is known about the regulation of Vi synthesis in vivo. However, the finding that Vi expression in *S. typhi* is regulated by members of the family of two-component systems, which are known to be important in controlling gene expression in vivo, coupled with what is known about its role in virulence suggests that there is a need to regulate this antigen in different host microenvironments in order for *S. typhi* to express full virulence.

It is interesting that natural infections with *S. typhi* and vaccination with live oral typhoid vaccines elicit poor Vi antibody responses even though it is known that Vi is a good immunogen and can be an effective vaccine when given as a subunit preparation (63). However, higher levels of Vi antibody are detected in individuals who become chronic carriers (63). It may be that the level of expression of this Vi polysaccharide is down regulated after the organism reaches its favored host environmental niche, i.e., an intracellular environment such as macrophages, because the organism no longer requires it for protection against the actions of the complement-mediated system. This may be an explanation for the poor Vi antibody responses elicited as only low doses of this antigen are presented to the immune system. We are at present investigating whether Vi antigen expression of *S. typhi* is reduced after invasion of epithelial cells and macrophages. We are also applying these findings to the possibility of developing more effective typhoid vaccines. It is well-known that live attenuated *Salmonella* vaccines elicit potent cell-mediated responses and can be very effective vaccines. Indeed, we have previously demonstrated that *ompR* mutants of *S. typhimurium* are excellent vaccines in mice. There is now some evidence that in order for live *Salmonella* vaccines to be effective, they must have the ability to elicit opsonizing antibody against the bacterial cell surface as well as eliciting cell-mediated immunity (43). Vi would therefore be a logical target against which to elicit opsonizing antibody for a typhoid vaccine. We are therefore constructing attenuated *S. typhi* strains that express Vi antigen constitutively or whose expression of Vi antigen is under the control of promoters that are activated in host intracellular compartments in order to determine whether the response against this antigen can be improved as a step to improving the efficacy of live oral typhoid vaccines.

ACKNOWLEDGMENT

We thank Tina Silva for all her hard work in the preparation of the manuscript.

REFERENCES

1. **Albright, L. M., E. Huala, and F. M. Ausubel.** 1989. Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. **23**:311–336.
2. **Aranda, C. M. A., J. A. Swanson, W. P. Loomis, and S. I. Miller.** 1992. *Salmonella typhimurium* activates virulence gene transcription within acidified macrophage phagosomes. Proc. Natl. Acad. Sci. USA **89**:10079–10083.
3. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. **175**:4475–4484.
4. **Bernardini, M. L., A. Fontaine, and P. J. Sansonetti.** 1990. The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. J. Bacteriol. **172**:6274–6281.
5. **Bernardini, M. L., M. G. Sanna, F. Fontaine, and P. J. Sansonetti.** 1993. OmpC is involved in invasion of epithelial cells by *Shigella flexneri*. Infect. Immun. **61**:3625–3635.
6. **Berry, A., D. DeVault, and A. M. Chakrabarty.** 1989. High osmolarity is a signal for enhanced *algD* transcription in mucoid and nonmucoid *Pseudomonas aeruginosa* strains. J. Bacteriol. **171**:2312–2317.
7. **Biggin, M. D., T. G. Gibson, and G. F. Hong.** 1983. Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA **80**:3963–3965.
8. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
9. **Chatfield, S., M. Roberts, P. Londono, I. Cropley, G. Douce, and G. Dougan.** 1993. The development of oral vaccines based on live attenuated *Salmonella* strains. FEMS Immunol. Med. Microbiol. **7**:1–8.
10. **Chatfield, S. N., C. J. Dorman, C. Hayward, and G. Dougan.** 1991. Role of *ompR*-dependent genes in *Salmonella typhimurium* virulence: mutants deficient in both *ompC* and *ompF* are attenuated in vivo. Infect. Immun. **59**:449–452.
11. **Chatfield, S. N., N. F. Fairweather, I. Charles, D. Pickard, M. Levine, D. Hone, M. Posada, R. A. Strugnell, and G. Dougan.** 1992. Construction of a genetically defined *Salmonella typhi* Ty2 *aroA aroC* mutant for the engineering of a candidate oral typhoid-

tetanus vaccine. Vaccine **10:**53–60.
12. **Chatfield, S. N., K. Strahan, D. Pickard, I. G. Charles, C. E. Hormaeche, and G. Dougan.** 1992. Evaluation of *Salmonella typhimurium* strains harbouring defined mutations in *htrA* and *aroA* in the murine salmonellosis model. Microb. Pathog. **12:**145–151.
13. **Deretic, V., R. Dikshit, W. M. Konyecshi, A. M. Chakrabarty, and T. K. Misra.** 1989. The *algR* gene, which regulates mucoidy in *Pseudomonas aeruginosa*, belongs to a class of environmentally responsive genes. J. Bacteriol. **171:**1278–1283.
14. **Deretic, V., J. F. Gill, and A. M. Chakrabarty.** 1987. *Pseudomonas aeruginosa* infections in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. Nucleic Acids Res. **15:**4567–4581.
15. **Deretic, V., C. D. Mohr, and D. W. Martin.** 1991. Mucoid *Pseudomonas aeruginosa* in cystic fibrosis: signal transduction and histone-like elements in the regulation of bacterial virulence. Mol. Microbiol. **5:**1577–1583.
16. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive selection suicide vector. Infect. Immun. **59:**4310–4317.
17. **Dorman, C. J.** 1991. DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infect. Immun. **59:**745–749.
18. **Dorman, C. J., S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan.** 1989. Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated in vivo. Infect. Immun. **57:**2136–2140.
19. **Dougan, G., S. Chatfield, D. Pickard, J. Bester, D. O'Callaghan, and D. Maskell.** 1988. Construction and characterisation of vaccine strains of salmonella harbouring mutations in two different *aro* genes. J. Infect. Dis. **158:**1329–1335.
20. **Filip, G., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium lauryl sarcosinate. J. Bacteriol. **115:**717–732.
21. **Foster, J. W., and H. K. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
22. **Galan, J. E., and R. E. Curtiss III.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
23. **Gottesman, S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. Mol. Microbiol. **5:**1599–1606.
24. **Govan, J. R. W.** 1988. Alginate biosynthesis and other unusual characteristics associated with the pathogenesis of *Pseudomonas aeruginosa* in cystic fibrosis, p. 67–96. *In* E. Griffiths, W. Donachie, and J. Stephen (ed.), Bacterial infections of respiratory and gastrointestinal mucosae. IRL Press, Oxford.
25. **Graeme-Cook, K. A., G. May, E. Bremer, and C. F. Higgins.** 1989. Osmotic regulation of porin expression: a role for DNA supercoiling. Mol. Microbiol. **3:**1287–1294.
26. **Hashimoto, Y., N. Li, H. Yokoyama, and T. Ezaki.** 1993. Complete nucleotide sequence and molecular characterization of ViaB region encoding Vi antigen in *Salmonella typhi*. J. Bacteriol. **175:**4456–4465.
27. **Heyde, M., and R. Portalier.** 1987. Regulation of major outer membrane porin proteins of *Escherichia coli* K12 by pH. Mol. Gen. Genet. **208:**511–517.
28. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–240.
29. **Hone, D. M., A. M. Harris, S. Chatfield, G. Dougan, and M. M. Levine.** 1991. Construction of genetically defined double *aro* mutants of *Salmonella typhi*. Vaccine **9:**810–816.
30. **Hornick, R. B., S. E. Greisman, T. E. Woodward, H. L. DuPont, A. T. Dawkins, and M. J. Snyder.** 1970. Typhoid fever: pathogenesis and immunologic control. N. Engl. J. Med. **283:**686–691.
31. **Houng, H. H.-S., K. F. Noon, J. T. Ou, and L. S. Baron.** 1992. Expression of Vi antigen in *Escherichia coli* K-12: characterization of ViaB from *Citrobacter freundii* and identity of ViaA with RcsB. J. Bacteriol. **174:**5910–5915.
32. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 of D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33:**933–938.
33. **Jayaratne, P., W. J. Keenleyside, P. R. MacLachlan, C. Dodgson, and C. Whitefield.** 1993. Characterization of *rcsB* and *rcsC* from *Escherichia coli* O9:K30:H12 and examination of the role of the *rcs* regulatory system in expression of group I capsular polysaccharides. J. Bacteriol. **175:**5384–5394.
34. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
35. **Jones, P. W., G. Dougan, C. Hayward, N. Mackensie, P. Collins, and S. N. Chatfield.** 1990. Oral vaccination of calves against experimental salmonellosis using a double *aro* mutant of *Salmonella typhimurium*. Vaccine **9:**29–36.
36. **Kaper, J. B., H. Lockman, M. M. Baldini, and M. M. Levine.** 1984. Recombinant non-toxinogenic *Vibrio cholerae* strains as attenuated cholera vaccine candidates. Nature (London) **308:**655–658.
37. **Kato, J., T. K. Misra, and A. M. Chakrabarty.** 1990. AlgR3, a protein resembling eukaryotic histone H1, regulates alginate synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **87:**2887–2891.
38. **Levine, M. M., and J. B. Kaper.** 1993. Live vaccines against cholera: an update. Vaccine **11:**207–212.
39. **Liljestrom, P., I. Laamanen, and E. T. Palva.** 1988. Structure and expression of the *ompB* operon, the regulatory locus for the outer membrane porin regulon in *Salmonella typhimurium* LT-2. J. Mol. Biol. **201:**663–673.
40. **Lindberg, A. A., A. Karnell, B. A. D. Stocker, S. Katakura, H. Sweiha, and F. P. Reinholt.** 1988. Development of an auxotrophic oral live *Shigella flexneri* vaccine. Vaccine **6:**146–150.
41. **Looney, R. J., and R. T. Steigbigel.** 1986. Role of the Vi antigen of *Salmonella typhi* in resistance to host defense *in vitro*. J. Lab. Clin. Med. **108:**506–516.
42. **Magasanik, B.** 1988. Reversible phosphorylation of an enhancer binding protein regulates the transcription of bacterial nitrogen utilization genes. Trends Biochem. Sci. **13:**475–479.
43. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect. Immun. **61:**3981–3984.
44. **Mekalanos, J. J.** 1991. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. **174:**1–7.
45. **Miller, S. I., and J. J. Mekalanos.** 1989. Strategies for the development of vaccines for typhoid fever, shigellosis, and cholera. Ann. N.Y. Acad. Sci. **569:**145–154.
46. **Miller, S. I., and J. J. Mekalanos.** 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. J. Bacteriol. **172:**2485–2489.
47. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
48. **Nakashima, K., K. Kanamaru, H. Aiba, and T. Mizuno.** 1991. Signal transduction and osmoregulation in *Escherichia coli*: a novel type of mutation in the phosphorylation domain of the activator protein, *ompR*, results in a defect in its phosphorylation-dependent DNA binding. J. Biol. Chem. **266:**10775–10780.
49. **NiBhriain, N., C. J. Dorman, and C. F. Higgins.** 1989. An overlap between osmotic and anaerobic stress responses: a potential role for DNA supercoiling in the coordinate regulation of gene expression. Mol. Microbiol. **3:**933–942.
50. **O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. F. Easmon, and G. Dougan.** 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attenuation, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419–426.
51. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
52. **Puente, J. L., A. Verdugo-Rodriguez, and E. Calva.** 1991. Expres-

sion of *Salmonella typhi* and *Escherichia coli* OmpC is influenced differently by medium osmolarity; dependence on *Escherichia coli* OmpR. Mol. Microbiol. **5**:1205–1210.

53. **Robbins, J. D., and J. B. Robbins.** 1984. Reexamination of the protective role of the capsular polysaccharide (Vi antigen) of *Salmonella typhi*. J. Infect. Dis. **150**:436–449.
54. **Russo, T. A., and G. Singh.** 1993. An extraintestinal, pathogenic isolate of *Escherichia coli* (O4/K54/H5) can produce a group 1 capsule which is divergently regulated from its constitutively produced group 2, K54 capsular polysaccharide. J. Bacteriol. **175**:7617–7623.
55. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
56. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
57. **Sansonetti, P. J., and J. Arondel.** 1989. Construction and evaluation of a double mutant of *Shigella flexneri* as a candidate for oral vaccination against shigellosis. Vaccine **7**:443–450.
58. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5**:1445–1454.
59. **Stephen, D., C. Jones, and J. P. Schofield.** 1990. A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. Nucleic Acids Res. **18**:7463–7464.
60. **Stewart, R. C., and F. W. Dahlquist.** 1987. Molecular components of bacterial chemotaxis. Chem. Rev. **87**:997–1025.
61. **Stock, J. P., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
62. **Tacket, C. O., D. M. Hone, R. Curtiss III, S. M. Kelly, G. Losonsky, L. Guers, A. M. Harris, R. Edelman, and M. M. Levine.** 1992. Comparison of the safety and immunogenicity of Δ*aroC* Δ*aroD* and Δcya Δcrp Salmonella typhi strains in adult volunteers. Infect. Immun. **60**:536–541.
63. **Tacket, C. O., G. Losonsky, D. N. Taylor, L. S. Baron, D. Kopecko, S. Cryz, and M. M. Levine.** 1991. Lack of immune response to the Vi component of a Vi-positive variant of the *Salmonella typhi* live oral vaccine strain Ty21a in human studies. J. Infect. Dis. **163**:901–904.
64. **Tartera, C., and E. S. Metcalf.** 1993. Osmolarity and growth phase overlap in regulation of *Salmonella typhi* adherence to and invasion of human intestinal cells. Infect. Immun. **61**:3084–3089.
65. **Tautz, D., and M. Renz.** 1983. An optimised freeze-squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. **132**:14–19.
66. **Wanner, B.** 1987. Phosphate regulation of gene expression in *Escherichia coli*, p. 1326–1333. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
67. **Wanner, B. L.** 1992. Is cross regulation by phosphorylation of two-component response regulator proteins important in bacteria? J. Bacteriol. **174**:2053–2058.
68. **Wozniak, D. J.** 1992. Involvement of integration host factor in the regulation of *Pseudomonas aeruginosa* alginate genes, *algB* and *algD*, abstr. D-232, p. 134. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
69. **Wozniak, D. J., and D. E. Ohman.** 1991. *Pseudomonas aeruginosa* AlgB, a two-component response regulator of the NtrC family, is required for *algD* transcription. J. Bacteriol. **173**:1406–1413.