INFECTION AND IMMUNITY, Nov. 1994, p. 4781–4788
0019-9567/94/$04.00+0

Vol. 62, No. 11

The Maltose Regulon of *Vibrio cholerae* Affects Production and Secretion of Virulence Factors

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Received 8 February 1994/Returned for modification 18 April 1994/Accepted 15 August 1994

The effects of maltose on production and secretion of virulence factors of *Vibrio cholerae* in strain X28214, classical biotype, and in maltose-defective transposon mutants constructed from this strain were characterized. Maltose was found to inhibit secretion of cholera toxin and to reduce production of the mannose-sensitive hemagglutinin and the soluble hemagglutinin-protease. In contrast, the amount of toxin-coregulated pilus was increased in the presence of maltose. The maltose effect was apparently mediated by genes of the maltose regulon, since inactivation of the *malQ* or *malF* gene of *V. cholerae* by transposon insertion was found to affect production and secretion of the same virulence factors that were responsive to maltose. The *malQ* and *malF* mutants showed, in addition, reduced virulence in an infant-mouse model. These results suggest that maltose may have a significant regulatory role in the production of virulence factors and that an intact maltose regulon is needed for full virulence of *V. cholerae*.

The gram-negative bacterium *Vibrio cholerae* O1 is the agent which causes cholera disease. Manifestation of the disease is the result of this organism's ability to colonize the small bowel and elaborate cholera toxin (CT), a complex protein composed of one A subunit (27 kDa) and five B subunits (11.6 kDa each). Cholera vibrios exist as two biotypes, designated classical and El Tor. The two biotypes have been observed to differ from each other not only in their preference for the culture conditions for optimal CT production but also in production of two different pilus structures. A toxin-coregulated pilus (TCP) has been shown to be an important colonization factor in classical strains (11, 35, 36), whereas the presence of a TCP on the cell surface of El Tor strains either in vivo or in vitro is still a matter of dispute (17) and its significance in colonization is uncertain (36, 39, 41). However, another type of pilus associated with mannose-sensitive hemagglutinin (MSHA) has been implicated as a potential adhesin of El Tor strains (16). In addition to the toxin and the pili, *V. cholerae* produces an arsenal of secreted proteins which are thought to assist vibrios in passage to the epithelial surface receptors through the mucus gel covering the intestine. A protease with mucinase activity, the soluble hemagglutinin-protease (SHA), has been suggested to be important in this respect (8).

Expression of the genes encoding CT and the TCP is coordinately regulated by the ToxR regulon, which is thought to be the main global regulatory system of *V. cholerae* involved in control of virulence factors (6, 29). However, the human gut provides several different environments that the vibrios encounter during their passage through their host. Accordingly, *V. cholerae* has to modulate its virulence gene expression in response to various environmental signals. Therefore, it is likely that other, still unidentified regulons are needed in addition to the ToxR regulon to assist in the fine-tuning and right timing of virulence gene expression that are required for a successful infection. We have previously shown that maltose causes alterations in the production of several outer membrane proteins, some of which are also controlled by the ToxR regulon (21, 22). In the process that leads to colonization of the intestine, the availability of nutrients is essential to *V. cholerae*, which could be reflected in alterations in outer membrane protein composition. Unfortunately, there is no available information on the preferred substrates of pathogens in the intestinal tract (23). For instance, sugar metabolism of *V. cholerae* and its role in virulence have not been characterized in any detail. Maltose could be an interesting sugar in this respect, as it is very common in the intestine and could provide a good substrate for the colonizing bacteria.

Very little is known about the role of maltose and maltose metabolism in *V. cholerae* pathogenesis. The maltose regulon in *Escherichia coli* has been extensively characterized (for a review, see reference 34). The maltose and maltodextrin uptake system is encoded by the *malEFG* and *malK-lamB-malM* operons, whereas the cytoplasmic maltodextrin-utilizing enzymes are encoded by the *malPQ* operon. These genes are all controlled by the transcriptional activator MalT, which is itself allosterically activated by maltose (32). The cyclic AMP receptor protein is also required for *mal* expression, linking the maltose regulon to the large family of catabolite-repressible genes, some of which are known to make a direct contribution to bacterial virulence (4, 9, 38).

We have recently characterized genes of the maltose regulon of *V. cholerae* by isolating maltose-defective transposon mutants of *V. cholerae* in which the transposon is located either in one of the maltose transport operons (*malF* mutants) or in maltose metabolism genes (*malQ* mutants) (20). These *mal* mutations affect production of the maltose-inducible maltoporin OmpS (22), which is analogous to LamB of *E. coli* (2), and other major outer membrane proteins of *V. cholerae* (21, 22). In this study, we have characterized the effects of maltose and *mal* mutations on the production and secretion of virulence factors CT, TCP, MSHA, and SHA. The results indicate that an intact maltose regulon is required for full virulence of

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TABLE 1. *V. cholerae* strains used in this study

Strain	Biotype or genotype	OmpS phenotype	Mal phenotype
Cairo 50	Classical Ogawa	Inducible	Mal^+
X28214	Classical Inaba	Inducible	Mal^+
HLX33	X28214 *toxR*::Km^r	Inducible	Mal^+
HLX481	X28214 *ompS*::Km^r	Negative	Mal^+
HLX511	X28214 *malF*::Km^r	Reduced	Mal^-
HLX531	X28214 *malQ*::Km^r	Constitutive	Mal^-

V. cholerae and that the production of these virulence factors is responsive to maltose.

MATERIALS AND METHODS

Bacterial strains and in vitro culture conditions. The *V. cholerae* strains used in this study are listed in Table 1. They were cultivated in L broth (pH 6.9), in CF broth (0.15% yeast extract, 1% Casamino Acids, 0.41 mM $MgSO_4$, 0.04 mM $MnCl_2$), or in M9 glycerol minimal medium containing the amino acids Asp, Arg, Glu, and Ser (25 mM each) in test tubes with constant shaking at 30°C. Maltose and glucose were added at 0.4%, and kanamycin was added at 25 μg ml^{-1}.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (19). The pH of the upper gel buffer was adjusted to 6.7 to increase the separation of outer membrane proteins in 12% acrylamide gels. Immunoblot analysis for detection of TcpA and MshA in whole-cell samples (3×10^6 bacteria) separated by SDS-PAGE was performed essentially as described elsewhere (17). Samples were boiled in the absence of 2-mercaptoethanol. The bovine serum albumin (BSA)-blocked nitrocellulose filters were first analyzed for the presence of TcpA by using anti-TCP monoclonal antibody (MAb) Tc 20:2 (15) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G as secondary antibodies (Jackson, West Grove, Pa.). H_2O_2 with 4-chloro-1-naphthol (Bio-Rad, Richmond, Calif.) was used as a substrate. Subsequently, and after several washings first in H_2O and then in phosphate-buffered saline (PBS), the same filters were scored for the presence of MshA with anti-MSHA MAb HA 17:10 (16). Alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibodies were used as secondary antibodies, and the reaction was developed by using Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (XP) (Boehringer GmbH, Mannheim, Germany) as substrates.

Inhibition ELISA for quantification of SHA. The production of SHA and the localization of SHA to cells and medium were determined from overnight cultures grown in CF broth by an inhibition enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody to SHA (40). In addition, all samples were tested for hemagglutination activity with chicken erythrocytes (40).

Toxin assay. The amount of CT in 1-ml samples of culture supernatants and in corresponding cell sonicates in 1 ml was measured by GM1 ganglioside-dependent ELISA (13) using a MAb against the B subunit and purified CT (List Biological Laboratories, Campbell, Pa.) as a reference. Sonicated cells were also assayed by dot blot analysis on nitrocellulose with MAb CT6, which recognizes both CT B-subunit (CTB) monomers and pentamers.

Inhibition ELISA for quantification of TCP. The relative amount of TCP on the cell surface was determined by an inhibition ELISA performed essentially as described previously (15), the major difference being the use of purified TCP (1.5 μg ml^{-1}) (26) diluted in 0.05 M carbonate buffer (pH 9.6) as a solid-phase antigen. The inhibition was performed in a separate, BSA-blocked plate; bacteria at an initial concentration of 8×10^9 cells per ml were serially diluted threefold in PBS containing 1% BSA and then mixed with an equal volume of anti-TCP MAb Tc 20:2. After incubation with gentle shaking at room temperature for 1 h, the reaction mixtures were transferred to the TCP-coated, washed plate and incubation was continued for 1.5 h. Horseradish-conjugated anti-mouse immunoglobulin G antibodies (Jackson) were used as secondary antibodies, and H_2O_2 and orthophenylene diamine were used as substrates. The interpolated dilution of bacteria causing inhibition of binding of specific anti-TCP antibody to the solid phase by 50% was determined.

Detection of MSHA on the cell surface. The effect of maltose on MSHA on the cell surface could not be assessed with strain X28214, since this strain lacks MSHA activity. Instead, another classical strain, Cairo 50, which is unusual in that it displays the El Tor-related MSHA on the cell surface, was used (18). Bacteria were grown at 37°C to mid-logarithmic phase in L broth in the presence or absence of 0.4% maltose. Washed bacteria were tested for slide agglutination with anti-MSHA MAb HA 17:10 as well as for binding to D-mannose covalently linked to agarose beads (18).

Binding to mannose-coated agarose beads and agglutination with antisera. The classical strain Cairo 50, which was grown in the absence or presence of 0.4% maltose, was tested for its ability to bind to mannose-coated agarose beads and for agglutination with an MSHA-specific MAb essentially as described by Jonson et al. (18).

Competition and virulence tests. In competition experiments, the competitive index was defined as the change in the ratio of two strains after growth together. The in vitro conditions were growth at 37°C for 20 h in L broth at a starting density of 5×10^4 CFU/ml. The in vivo conditions were intraintestinal growth in 3- to 5-day-old suckling mice inoculated orally with 5×10^4 CFU and sacrificed 24 h later. The input ratio was approximately 1 in both in vitro and in vivo competitions. Viable cells were recovered and counted by plating dilutions of broth and intestinal homogenate on L agar and *Vibrio*-selective thiosulfate-citrate-bile salts-sucrose agar (TCBS) plates, respectively. The ratio of the two strains was determined by scoring the colonies for Km^r. The in vivo competition index is the average of values obtained from three individual mouse experiments. Virulence was tested by the infant-mouse assay, which was performed essentially as described by Osek et al. (27). Each mouse was inoculated orally with 2.5×10^7 CFU in 50 μl of PBS. The condition of the mice was examined after 48 h.

RESULTS

Maltose inhibits secretion of CT. Several environmental signals have been implicated in CT production (1, 25). However, the factors affecting secretion of CT to the extracellular milieu are less well characterized. As maltose is known to cause profound changes in the outer membrane protein composition (21, 22), we wanted to investigate whether it also would promote changes in secretion of virulence factors across the outer membrane. The classical strain X28214 was grown under conditions promoting high levels of toxin production, and the amounts of CT in culture supernatants and cells were determined by GM1 ELISA at three time points (3, 6, and 17 h). The results (Fig. 1) show that in the absence of maltose

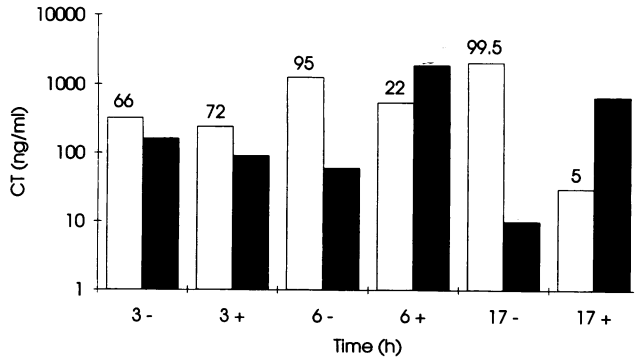

FIG. 1. Amounts of CT in X28214 cells (filled bars) and culture supernatants (open bars). The cells were grown in 5 ml of L broth (pH 6.9) in test tubes with constant shaking at 30°C. Samples were taken at 3, 6, and 17 h, and the amounts of CT were measured by GM1 ELISA. Cultures were grown in the absence (−) and presence (+) of 0.4% maltose. Numbers above the bars indicate percentages of toxin secreted to the supernatant.

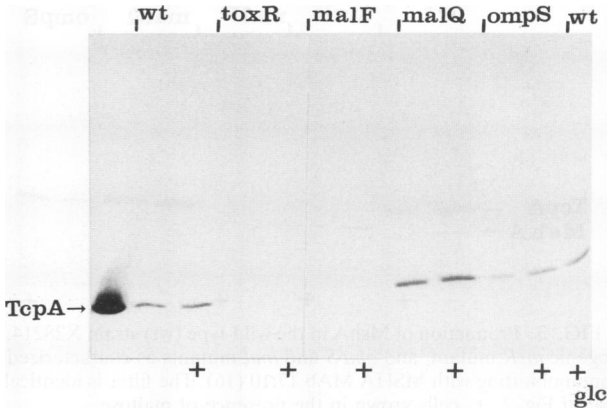

FIG. 2. Characterization of TcpA from the wild-type (wt) strain X28214, an isogenic *toxR* mutant, and *ompS* and *mal* mutants by Western blotting (immunoblotting). The *V. cholerae* strains were grown overnight in CF broth at 30°C. TcpA was identified with TcpA MAb 20:2 (15). Cells were grown in the presence of 0.4% maltose (+) or in the presence of 0.4% glucose (glc) as indicated.

more than 95 and 99% of the toxin was secreted into the growth medium at 6 and 17 h, respectively. In contrast, the presence of maltose inhibited the secretion of CT into the extracellular milieu, and this effect was obvious already 6 h after maltose addition, when 78% of the total toxin was cell associated compared with 5% in the control. An even more dramatic change in toxin distribution between cells and supernatant was observed at 17 h, when 95% of the toxin was associated with maltose-grown cells whereas only 0.5% was found in the control cells. At 3 h, no significant difference in the distribution of CT between the cultures grown in the absence and presence of maltose was observed. There was no major difference in the amount of toxin produced in the presence and absence of maltose until 17 h, when the total amount of toxin was smaller in the cells grown in the presence of maltose. A similar decrease in total toxin levels in maltose-grown stationary-phase cells was also evident in other experiments (see Table 4). The cell-associated CT assayed by GM1 ELISA was most likely localized to the periplasm, since the method detects only assembled B-subunit pentamers, which are formed first in the periplasm (12). We did not detect any marked accumulation of CTB monomers by using a MAb recognizing such monomers (not shown), but the cell-bound CT appeared to be in the pentamer form.

Maltose induces TCP. The dramatic effect of maltose on secretion of CT prompted us to study its effect on other virulence determinants. Production of the TCP of *V. cholerae* has been shown to follow the same regulatory pattern as CT (42). The relative amounts of TCP on the cell surfaces of the wild-type strain X28214 and of an isogenic *toxR* mutant, HLX33, were determined by TCP inhibition ELISA, and the amounts of the structural subunit, TcpA, were estimated by immunoblotting of whole cells with a MAb (Tc 20:2) against TCP (Table 2; Fig. 2). Surprisingly, the results from the inhibition ELISA indicate that, in contrast to the observed inhibition of CT production and secretion, the amount of TCP located on the cell surface was increased approximately 10-fold by maltose in the wild-type strain. When the cells were grown in minimal medium, the total amount of TcpA was also somewhat increased with maltose (Table 2). The *toxR* mutant used as a control did not produce detectable amounts of TcpA.

Maltose affects two putative virulence factors, the cell-associated MSHA and SHA. MSHA is a potential virulence factor of El Tor vibrios that has been shown to be associated with a pilus structure and that is also present on El Tor vibrios grown in rabbit and human intestines (16). Only a few classical strains have been found to display MSHA on the cell surface, although the structural subunit of the pilus, MshA, has been detected in all classical strains tested (16). Since strain X28214

TABLE 2. Amounts of TcpA on the cell surface and in whole-cell samples of *V. cholerae*[a]

Strain	Genotype	TCP (ELISA)[b]			Result of TcpA immunoblot[c]			
					In CF broth		In M9 glycerol	
		Without maltose	With maltose	With maltose and glucose	Without maltose	With maltose	Without maltose	With maltose
X28214	wt[d]	1	12	12	+++	+++	++	+++
HLX33	*toxR*	<1	<1		−	−	−	−
HLX481	*ompS*	<1	10		+	++	+++	++++
HLX511	*malF*	<1	<1		−	−	+/−	+
HLX531	*malQ*	0.5	1		++++	+++++	+	++

[a] Cells were grown in the absence or presence of 0.4% maltose and 0.4% glucose as indicated.
[b] The interpolated dilution of bacteria, grown in CF broth for 16 h, causing 50% inhibition of binding of specific anti-TCP MAb to the solid phase by TCP.
[c] The relative amount of TcpA determined visually from immunoblots with MAb Tc 20:2. −, no reaction; +/−, faintly visible reaction; +, clearly visible (number of pluses indicates intensity of the reaction).
[d] wt, wild type.

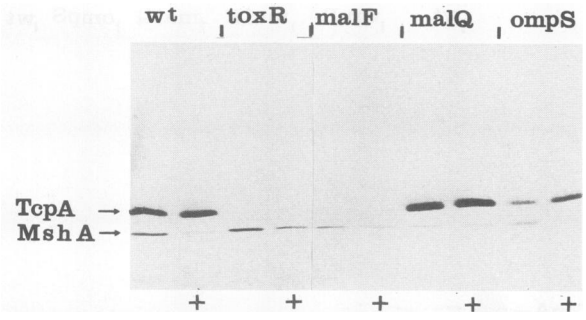

FIG. 3. Production of MshA in the wild-type (wt) strain X28214, an isogenic *toxR* mutant, and *ompS* and *mal* mutants as characterized by immunoblotting with MSHA MAb 17:10 (16). The filter is identical to that of Fig. 2. +, cells grown in the presence of maltose.

is MSHA negative, the effect of maltose on production of the pilus subunit MshA was assessed from this strain and from an isogenic *toxR* mutant by using an MSHA-MshA-reactive MAb. Immunoblotting of the same bacterial samples that had been scored for TcpA production shows (Fig. 3) that the production of MshA was reduced in the presence of maltose. Interestingly, there was no difference in MshA production between the *toxR* mutant and the wild type, providing evidence that synthesis of the structural subunit of MSHA is independent of ToxR.

In view of the profound effect of maltose on the surface localization of TCP, we also examined whether the amount of MSHA on the cell surface is affected by maltose. The classical strain Cairo 50, which is MSHA positive, after growth in maltose-containing medium, was found to be negative in both the mannose-agarose binding test and the agglutination test with the MSHA-specific MAb (data not shown). The control bacteria were strongly positive in both tests. In conclusion, maltose apparently has opposite effects on the production and translocation across the outer membrane of the two pili, TCP and the MSHA-pilus.

SHA has been implicated as an important virulence factor in *V. cholerae*, since the protease and mucinase activities of SHA may enhance the ability of the vibrios to penetrate the mucus covering the intestinal epithelium (3, 8). The concentration of SHA in the culture supernatant was reduced drastically when the wild-type strain X28214 was grown in the presence of maltose (Table 3). Cell-associated SHA was also examined but was not detectable. Thus, most, if not all, of the residual SHA was secreted to the medium also in the presence of maltose, suggesting that the production of SHA was strongly inhibited by maltose.

In conclusion, maltose appears to have an inhibitory effect on production or secretion, or both, of several virulence factors such as SHA, CT, and MSHA but in contrast appears to facilitate secretion of others such as TCP.

TABLE 3. Effect of maltose on secreted SHA in X28214 and *mal* mutants[a]

Strain	Genotype	SHA in supernatant (ng/ml)	
		Without maltose	With maltose
X28214	wt[b]	2,000	<100
HLX33	*toxR*	500	<100
HLX481	*ompS*	300	<100
HLX511	*malF*	100	<100
HLX531	*malQ*	<100	<100

[a] Cells were grown in CF broth at 30°C for 12 h without or with the addition of 0.4% maltose as indicated.
[b] wt, wild type.

TABLE 4. Effect of maltose on the amounts of cell-bound and secreted CT in *mal* mutants of *V. cholerae*[a]

Strain	Genotype	CT (ng/ml)			
		In supernatant		In cells	
		Without maltose	With maltose	Without maltose	With maltose
X28214	wt[b]	>5,000	30	76	377
HLX33	*toxR*	1	0.2	1	0.2
HLX481	*ompS*	939	30	1,180	684
HLX511	*malF*	7	26	5	32
HLX531	*malQ*	2,220	3,680	2,390	3,440

[a] Bacteria were grown in CF broth for 16 h in the absence or presence of 0.4% maltose as indicated.
[b] wt, wild type.

mal mutants of *V. cholerae* are affected in CT production and secretion. As maltose was found to have a substantial effect on secretion and production of virulence factors, we wanted to examine whether this effect would be mediated by genes of the maltose regulon. To this aim, we employed *mal* mutants carrying transposon insertions in the *ompS*, *malQ*, or *malF* gene of *V. cholerae* (20). The major characteristics of the mutants are listed in Table 1. The *ompS* gene encodes the outer membrane maltoporin of *V. cholerae* (22), while *malF* defines a component of the maltose transport system and *malQ* encodes an enzyme in the maltose metabolism (20, 34). The *malF* mutant shows reduced expression of *ompS*, while *malQ* mutants express this gene constitutively (20). These mutants and, for comparison, a wild-type strain and a corresponding *toxR* mutant were first used to measure the toxin concentration in culture supernatants and bacterial cells by GM1 ELISA (Table 4). In the wild type (X28214), maltose prevented the secretion of CT as expected (cf. Fig. 1); the amount of secreted toxin was reduced >160-fold, from >5,000 to 30 ng ml^{-1}, by the presence of maltose. The *toxR* mutant (HLX33) produced very small amounts of toxin, but still maltose inhibition of toxin production was suggested.

The total amount of CT detected in the *malF* mutant was drastically reduced compared with the values for the wild type, indicating that the production of holotoxin was affected in these mutants (Table 4). We could not find any intracellular accumulation of CTB monomers in the *malF* mutant, using a MAb that reacts with both CTB monomers and pentamers. The amount of monomers detected was directly proportional to the amount of cell-associated pentameric CTB, suggesting that the mutation may directly affect production of CTB monomers. In contrast, the *malQ* mutant showed no reduction in the total amount of toxin. Furthermore, the distribution of CT in this mutant was not responsive to maltose. The *ompS* mutant produced somewhat less toxin than the wild type and was affected in its secretion. In the presence of maltose, the distribution of CT was similar to that of the wild type, i.e., the majority of the toxin was associated with the cells. In conclusion, all the *mal* mutants tested were affected in their ability to either produce or secrete CT.

TcpA production is affected in _mal_ mutants. To test whether the maltose-induced increase in the amount of TCP on the cell surface was mediated by *mal* genes, we measured the production of TCP and TcpA in these mutants, using the same methods as described for the wild type (Table 2; Fig. 2).

TABLE 5. Characterization of the wild type (X28214) and *ompS* and *mal* mutants in infant-mouse intestines

Strain	Genotype	No. of surviving mice/ total		Diarrhea[a]
		Expt 1	Expt 2	
X28214	wt[b]	0/5	6/13	++
HLX481	*ompS*	2/5		+
HLX511	*malF*	3/4[c]	12/13[d]	−
HLX531	*malQ*	3/4[c]	14/14[e]	+

[a] −, none; +, moderate; ++, severe.
[b] wt, wild type.
[c] $P = 0.048$.
[d] $P = 0.015$.
[e] $P = 0.0019$.

Results from the TCP inhibition ELISA indicated that only small amounts of TCP were present on the cell surface of *malF* mutants and that maltose no longer induced TCP. Furthermore, even the production of the TcpA subunit protein was strongly reduced. The *malQ* mutant, which is OmpS constitutive, possessed an interesting phenotype: the total amount of TcpA was increased, but there was very little TCP detectable on the cell surface. In conclusion, the *malF* and *malQ* mutants were impaired in their ability to produce a functional TCP.

MshA and SHA are affected in *mal* mutants. Results from immunoblotting indicated that, similarly to the results for the wild type, maltose reduced the amount of MshA produced by the *ompS* and *malF* mutants (Fig. 3). On the other hand, the *malQ* mutants, which are impaired in maltose metabolism, exhibited a low-level constitutive production of MshA. This suggests that the *malQ* gene could be involved in mediating the effect of maltose on MshA production.

Similar to the results with MshA, maltose caused a clear but less dramatic reduction in the amount of SHA in the *ompS* and *malF* mutants than that observed in the wild type (Table 3), suggesting that the mutations could affect the production of SHA.

***mal* mutants are less virulent in the infant-mouse model than the wild-type strain.** The inhibitory effects of *mal* mutations on CT and TCP production and/or secretion prompted us to investigate the virulence of these mutants in the infant-mouse model. First, four or five mice were infected orally with each strain with a dose of bacteria that according to earlier tests gave 90 to 100% mortality in 3-day-old mice for strain X28214 (2.5×10^7 CFU), and the mice were inspected for diarrhea and mortality after 48 h. The mortality rates caused by the mutants were compared with that of the wild type. As expected, none of the mice infected with the wild-type control survived, but three of the four mice infected with the *malF* or *malQ* strain survived ($P = 0.048$, Fisher's exact test) (Table 5, experiment 1). In addition, mice challenged with the *malF* mutant did not show any signs of diarrhea, as the control mice did. These results suggest that the *malF* and *malQ* mutants are reduced in virulence. Although the animals used in a second experiment were less sensitive, probably because they were older, the impaired virulence of these two mutants was confirmed (Table 5, experiment 2). The *P* values obtained with Fisher's exact test were 0.015 and 0.0019 for *malF* and *malQ*, respectively. The presence of surviving mice in the group challenged with the *ompS* mutant suggests that this strain may also have reduced virulence, but the sample size was too small to determine if this reduction was significant.

***ompS* mutant is not outcompeted by the wild type in the infant-mouse model.** We have previously suggested that OmpS protein could play a dual role in *V. cholerae* infection: it could be important early in infection in uptake of maltose and later in uptake of other carbohydrates under nutrient-limiting conditions (22). To test this and to clarify the role of *ompS* in virulence (see above), the *ompS* mutant HLX481 was assessed in a competition assay against the wild type by infecting mice with 5×10^4 bacteria containing the *ompS* mutant and the wild type in a 1/1 ratio and calculating the ratio of the two strains in the intestine after 24 h. Both the mutant and the wild type multiplied in mice (to total 8×10^7 bacteria per mouse), and the ratio was not significantly changed. The competition indices of HLX481 were calculated to be 0.57 in vivo and 0.89 in vitro. This suggests that the *ompS* mutant is not dramatically altered in its colonizing capacity compared with the wild-type strain in the infant-mouse model.

DISCUSSION

In this study, we have characterized the effect of maltose on production and secretion of CT, TCP, MSHA, and SHA in the *V. cholerae* O1 wild type and in different types of *mal* transposon mutants. The results show that maltose has a profound effect both on virulence factors that are secreted into the extracellular milieu and on pilus proteins associated with the bacterial cell surface. In the wild-type strain, maltose markedly reduced the amount of secreted CT and SHA. The amount of MshA, the structural subunit of MSHA, was reduced, and the display of MSHA on the bacterial surface was abolished. In contrast, TCP on the cell surface was induced 10-fold when the cells were grown in the presence of maltose. In most cases, the effects are specific for maltose (our unpublished observation). However, glucose may act, possibly via catabolic repression, on production of, e.g., MshA and also on secretion of CT (data not shown). Glucose also reduced the amount of TcpA (Fig. 2), in contrast to the increase observed with maltose. In addition, glucose seems to inhibit the growth of *V. cholerae* in vitro when the cells in rich medium enter the stationary phase. Similar inhibition is not seen in cells grown in the presence of maltose. The effect of glucose is probably not significant in vivo, as there is a very limited amount of free glucose available in the intestine. We also confirmed that the effect of maltose and glucose together was the same as that obtained with maltose alone, indicating that the effects we saw were not due only to growth phase or to the physiological state of the cells.

Apparently, genes of the maltose regulon are mediating at least some of the observed effects of maltose. As the *malF* strain is a maltose transport mutant (20, 37), the low levels of CT and monomeric CTB detected may suggest that synthesis of CT is compromised when the maltose transport system is defective. The maltose transport system may also interact with a component that is needed for biogenesis of TCP. Thus, an intact maltose transport system seems to be crucial for translocation of virulence factors across the outer membrane. Furthermore, the *malF* mutant is affected in production of TcpA, suggesting that maltose could be involved in regulation of expression of virulence genes, e.g., *tcpA*.

The *malQ* mutant involved in maltose metabolism (20) seems to express the maltose regulon constitutively and produce large amounts of toxin, secretion of which is no longer responsive to maltose inhibition. The mutant also produced larger amounts of cell-associated TcpA. *E. coli malQ* mutants are defective in amylomaltase production and accumulate internally synthesized maltose and maltotriose, which are responsible for the constitutive nature of these mutants (5). A similar kind of internal formation of malto-oligosaccharides

could be responsible for the effect on virulence factors in *malQ* mutants of *V. cholerae*. It is interesting that the phenotype of this strain resembles somewhat that of 569B, which shows constitutive production of toxin, TCP (24), and OmpS (21). However, we cannot rule out the possibility that the phenotype of the *malQ* mutant is due to a polar effect on other genes or that the *malQ* gene product, amylomaltase, is interacting with a gene product of *V. cholerae* that is involved in production or secretion of virulence factors.

The mutant carrying a transposon insertion in the *ompS* gene was not significantly affected in the production or secretion of other virulence factors except for CT. When grown without maltose, it retained more toxin in the cells than the corresponding wild-type strain. This suggests that the OmpS protein in the outer membrane might affect secretion of CT. The lack of OmpS could compromise the structural integrity of the outer membrane and consequently affect proteins associated with it, including those that are needed to assist in CT secretion.

Further evidence for the role of the maltose regulon in control of virulence of *V. cholerae* was provided by results from the infant-mouse virulence test in which two of the *mal* mutants were clearly attenuated. A defect in the maltose transport system seems to have important implications in this respect, as the *malF* mutant, impaired in the production of both CT and TCP, was one of the mutants found to be practically avirulent. The *malQ* mutant was also less virulent than the wild type. This indicates that constitutive production of virulence factors can also result in impaired virulence. The *ompS* mutant was tested also by competition assay and found to successfully compete with the wild-type strain in mice. It seems not to be severely affected in TCP and CT production and/or secretion or in virulence, which correlated well with the notion that these factors are important for virulence in *V. cholerae*.

What would be the mechanism by which maltose affects *V. cholerae* virulence factors? There is no indication from our previous studies that the maltose regulon of *V. cholerae* directly interacts with the ToxR regulon or with other virulence regulons at the transcriptional level (20, 22). Rather, maltose and the maltose transport system may interact with components required for secretion of virulence factors. Common components seem to be employed for translocation of several proteins across the outer membrane, including a class of global periplasmic thiol-disulfide interchange proteins that participate in disulfide bond formation of periplasmic and outer membrane proteins (for a review, see reference 30). In *V. cholerae*, TcpG, which is required for the functional maturation of TCP, also has a role in secretion of a protease that probably is identical to SHA (28). Yu et al. (44) have identified a gene, *dsbA*, from *V. cholerae* which encodes a protein identical to TcpG that is required for biogenesis of CT. Cytoplasmic proteins containing an ATP-binding domain (Walker boxA) (43) function in extracellular protein secretion and assembly of type IV pili. Sandkvist et al. (33) have described a similar protein from *V. cholerae*, EpsE, which seems to be important for CT secretion. DNA encoding EpsE as well as other products homologous to the secretory factors encoded by the pullulanase gene cluster in *Klebsiella* spp. (31) has also been found in strain X28214 (14). The pleiotropic effects of *mal* mutations could be explained by assuming that there are common elements in the secretory machineries for virulence factors and that the maltose transport system may directly interact with these proteins that are needed for, e.g., TCP and CT biogenesis. Alternatively, maltose may induce some of the corresponding gene clusters and repress others. Thus, maltose could affect some component in the terminal branch of the general secretory pathway in *V. cholerae* that helps to secrete some virulence factors (TCP) and represses secretion of other factors (CT, MSHA, and SHA). Whether maltose affects TcpG and EpsE, the two identified proteins that are involved in secretion of extracellular proteins, remains to be elucidated.

To be a successful pathogen, *V. cholerae* must be able to regulate its virulence factors in response to the signals generated in the different microenvironments in the human gut. Since growth, colonization, and detachment from epithelial cells all require the action of different gene products, a complex regulatory network is probably involved in controlling virulence factors in *V. cholerae*. On the basis of the results obtained with the *mal* mutants and the effect of maltose on secreted virulence factors in wild-type *V. cholerae*, we suggest that maltose could be an early signal in pathogenesis leading to induction of TCP and repression of CT secretion. Almost all of the starch we eat is in the form of maltose and maltodextrins when it enters the duodenum from the stomach. This could provide *V. cholerae* with nutrients, enabling the bacteria to multiply. The maltose could also be a signal telling the vibrios that they have arrived in an environment where they can produce the colonization factors required for the early stage of adherence, the attachment to the mucus layer. Maltose could be an important signal in the correct timing of virulence gene expression. The production of adhesins enabling the vibrios to attach, individually or in aggregates, to the mucosal surface would be important at the early stage of infection. In accordance with this hypothesis, we find that TCP, which is known to cause autoaggregation of vibrios (42), is induced with maltose. In contrast, the timing of MSHA expression may be different, as suggested by its inhibition by maltose in an MSHA-positive classical strain. Later during infection, when the concentration of maltose in the vicinity of the vibrios decreases, the production of TCP may cease, and mucinases and motility, which help the vibrios to traverse the mucus gel, may be induced. With the help of chemotaxis, vibrios arrive at the epithelial cell surface, where they may produce other adhesins, multiply, and secrete CT in proximity to the GM1 receptors. At this stage, an additional role of SHA-protease, which was found to be repressed by maltose, in detachment has been postulated (7). This model may explain the weak antibody responses against TCP in convalescents and volunteers infected with *V. cholerae* (10). Interestingly, the classical MSHA-positive strain Cairo 50 lacks TCP (15) but instead produces a fucose-sensitive hemagglutinin, which we have found in preliminary experiments to be increased in the presence of maltose (data not shown). Thus, it may be speculated that similarly to TCP, fucose-sensitive hemagglutinin may be an adhesin involved in the early colonization of the intestine.

In conclusion, the results of this study demonstrate that maltose and *mal* mutations have a profound effect not only on outer membrane proteins but also on CT secretion and/or production and on the number of pilus structures on the cell surface. These results can be understood in context of a simple hypothesis. (i) A maltose-derived sugar molecule (malto-inducer) induces TcpA and CT production and represses the production of MshA and SHA. (ii) The malto-inducer activates biogenesis of TCP and blocks secretion of CT. (iii) An intact maltose transport system is required for this control. (iv) *malQ* of *V. cholerae* is involved in synthesis and breakdown of this malto-inducer.

ACKNOWLEDGMENTS

We thank Jacek Osek for valuable help with the virulence tests and Robert Andersson for the completion of the revised version of the manuscript.

This work was supported by the Swedish Medical Research Council (grants 07487 and 16X-3382) and the Faculty of Medicine, University of Göteborg.

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