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Mutants Carrying Conditionally Lethal Mutations in Outer Membrane Genes *omsA* and *firA* (*ssc*) Are Phenotypically Similar, and *omsA* is Allelic to *firA*

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We have previously identified the gene (the *ssc* gene) defective in the thermosensitive and antibiotic-supersusceptible outer membrane permeability mutant SS-C of *Salmonella typhimurium* and shown that this gene is analogous to the *Escherichia coli* gene *firA* (L. Hirvas, P. Koski, and M. Vaara, EMBO J. 10:1017–1023, 1991). Others have tentatively implicated *firA* in a different function, mRNA synthesis. Here we report that the defect in the thermosensitive outer membrane *omsA* mutant of *E. coli* (T. Tsuruoka, M. Ito, S. Tomioka, A. Hirata, and M. Matsuhashi, J. Bacteriol. 170:5229–5235, 1988) is due to a mutation in *firA*; this mutation changed codon 271 from serine to asparagine. The *omsA*-induced phenotype was completely reverted by plasmids containing wild-type *firA* or *ssc*. Plasmids carrying the *omsA* allele, or an identical mutant allele prepared by localized mutagenesis, under the control of *lac* elicited partial complementation. Transcomplementation studies with plasmids carrying various mutant alleles of the *S. typhimurium* gene indicated that the ability of these plasmids to complement the *omsA* mutation was similar to their ability to complement the *ssc* mutation. The antibiotic-supersusceptible phenotype of the *omsA* mutant closely resembled that of the *ssc* mutant, i.e., the *omsA* mutant was supersusceptible to hydrophobic antibiotics and large-peptide antibiotics against which the intact outer membrane is an effective permeability barrier. As previously demonstrated with the *omsA* mutant, the outer membrane of the *ssc* mutant became selectively ruptured after incubation for 1 h at the growth-nonpermitting temperature; 82% of the periplasmic β-lactamase and less than 3% of the cytoplasmic marker enzyme were released into the medium. All of these findings are consistent with our concept that *firA* is an essential gene involved in generation of the outer membrane.

The outer membrane (OM) of gram-negative bacteria is one of the most comprehensively characterized biological membranes (26, 31). However, we still lack the complete picture of its biosynthesis. The translocation process of OM proteins is only partially known (49). Very little is known about export of the lipopolysaccharide (LPS) component from its synthesis site in the cytoplasmic membrane to the outer leaflet of the OM (33). Furthermore, even though lipid A is the essential part of LPS for viability of bacterial cells, only 2 (*lpxA* and *lpxB*) of the approximately 8 to 10 genes involved in its biosynthesis have been discovered (33). The search for the remaining essential genes of OM biogenesis has been hampered by the lack of mutants conditionally defective in such genes.

A characteristic feature of the intact enterobacterial OM is its relative impermeability to hydrophobic drugs (28–30). The impermeability is due to LPS (29, 45); the outer leaflet of the OM contains LPS as its only phospholipid and lacks glycerophospholipid patches, the effective channels for hydrophobic diffusion (29–31). Agents that damage the OM sensitize the bacteria drastically to hydrophobic inhibitors (40). Also, mutants which have severe defects in the nonessential inner carbohydrate core region of LPS (chemotypes Re, Rd_2, Rd_1, and RcP^-) are supersusceptible (14, 28, 30, 37). Therefore, supersusceptibility to hydrophobic drugs may suggest a defect in the OM.

Accordingly, novel mutants which are conditionally lethal and, under permissive conditions, supersusceptible to hydrophobic antibiotics could be mutated in previously unknown essential OM genes and, hence, could turn out to be invaluable in elucidation of the remaining enigmas of OM biogenesis. Such mutants include the *omsA* (outer membrane structure) mutant of *Escherichia coli* (39) and the *ssc-1* (supersensitive class C) mutant of *Salmonella typhimurium* (17, 38). Both have been shown to be extremely susceptible to a wide set of such antibiotics, against which the OM is an effective permeability barrier. Both are unable to grow at 42°C. The study on the *ssc-1* mutation led us to discover and sequence a novel gene of *S. typhimurium*, the *ssc* gene (16). The sequence of its analog in *E. coli*, the *firA* gene, is also known (5). On the other hand, the gene mutated in the *omsA* mutant was not identified by Tsuruoka et al. (39), but their preliminary evidence suggested that the *omsA* mutation maps at approximately 4 min on the *E. coli* chromosome. Since the *firA* gene is also located at 4 min, we felt it necessary to study the possible relationship of these genes. In this report, we show that the *omsA* mutation is allelic to *firA*. Furthermore, we show that the phenotypic characteristics of the *ssc-1* (*firA*) mutant of *S. typhimurium* and the *omsA* mutant of *E. coli* closely resemble each other.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* and *S. typhimurium* strains and plasmids used are described in Table 1. The *E. coli* cloning hosts were JM105 (50) for vector pUC19 (50), TG1 (8) for phage M13mp8 or M13mp9 (27), and XL1-Blue (2) for Bluescript SK+ (1). Cultures were grown in L broth (17) or 2× TY medium (34), supplemented with ampicillin (100 μg/ml) or ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml) when appropriate.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant property	Reference(s)
E. coli		
CDH23-210	HfrC *metB1 proA3 relA1 lac-3 malA36 tsx-76* K-12	39
CDH23-213	*omsA* sister transductant of CDH23-210	39
S. typhimurium		
SH5014	*ilv-1178 thr-914 his-6116 metA22 metE551 trpB2 xyl-404 H1-b H2-e,n,x flaA66 rpsL120 rfaJ4041* LT2	36
SH7622	*ssc-1* (from SH5014)	17, 38
Plasmids		
pUCHS16A[a]	*ompH*$^+$	17
pUCHS14-II[a]	*ssc*$^+$	17
pSSC101[a]	Encodes Ssc with amino acid substitution Val-291→Met	19
pSSC102[a]	Encodes Ssc with amino acid substitution Met-288→Leu	19
pSSC103[a]	Encodes Ssc with amino acid substitution Met-290→Leu	19
pSSC104[a]	Encodes Ssc with amino acid substitution Met-292→Leu	19
pSSC105[a]	Encodes Ssc with amino acid substitution Gly-27→Asp	19
pSSC106[a]	Encodes Ssc with amino acid substitution Met-31→Leu	19
pSSC107[a]	Encodes Ssc with amino acid substitution Leu-130→Met	19
pSSC108[a]	Encodes Ssc with amino acid substitution Gly-131→Asp	19
pSSC109[a]	Encodes Ssc with amino acid substitution Met-288→Lys	19
pSSC110[a]	Encodes Ssc with amino acid substitution Met-290→Lys	19
pSSC111[a]	Encodes Ssc with amino acid substitution Met-292→Lys	19
pSSC112[a]	Encodes Ssc with amino acid substitution Gly-289→Asp	19
pGAH317	*hlpA*$^+$ *firA*$^+$	20
pOMSA101[b]	*omsA*	This work
pOMSA102[b]	*omsA*	This work
pOMSA103[b]	*firA*$^+$	This work

[a] The *lac* promoter of the vector is opposite in orientation to the *ompH* or *ssc* gene.

[b] The *lac* promoter of the vector is upstream and in the same orientation as the *firA* gene. The wild-type *firA* gene was cloned from pGAH317, and the *omsA* allele was cloned from CDH23-213. Plasmid pOMSA102 contains the *omsA* allele made by site-directed mutagenesis.

DNA techniques. General DNA manipulations were carried out as described by Sambrook et al. (34). Isolation of chromosomal DNA was done by the modified method of Marmur as described previously (22). Nucleotide sequences were determined by the chain termination method (35) with Sequenase (United States Biochemical, Cleveland, Ohio). Site-directed mutagenesis was done by using the oligonucleotide mutagenesis kit, version 2 (Amersham, Buckinghamshire, England), and Bluescript SK+ (Stratagene, La Jolla, Calif.) as the vector.

Southern hybridization. Southern blotting was done under alkaline conditions with positively charged nylon membranes (Boehringer, Mannheim, Germany). The filter was prehybridized with 20 ml of hybridization buffer (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 2% blocking reagent [catalog no. 1096176; Boehringer], 0.1% *N*-lauroylsarcosine, 0.02% sodium dodecyl sulfate [SDS]) for 1 h at 42°C. Hybridization was performed with 2.5 ml of the same solution and 500 pmol of the 30-mer *firA*-specific oligonucleotide probe CTGGCTGATT TAGCGCAGCAGTTGGATGCA labeled by digoxigenin-dUTP tailing (Boehringer) for 18 h at 42°C. The filter was washed twice for 5 min each time with 2× SSC–0.1% SDS at room temperature and then twice for 5 min each time with 0.1× SSC–0.1% SDS at 54°C. Detection was performed with a DIG luminescence detection kit (Boehringer) as instructed by the supplier.

Growth and viability determinations. Growth was measured as the A_{550}, as well as by determining the viable count by plating on L agar.

Antibiotic susceptibility tests. MICs were determined by the broth microdilution method in L broth. The inoculum was 10^4 L agar-grown cells per ml. Incubation was for 18 h at 28 or 39°C. The antibiotics were fusidic acid (Na salt), rifampin, clindamycin hydrochloride, and bacitracin (65,000 U/mg; Sigma Chemical Co., St. Louis, Mo.), erythromycin ethyl succinate (Orion, Helsinki, Finland), vancomycin hydrochloride (Eli Lilly & Co., Indianapolis, Ind.), and mupirocin (SmithKline Beecham, Worthing, West Sussex, England). As indicated elsewhere in the text, an alternative MIC test (E Test; AB Biodisk, Solna, Sweden) (42) was used. Bacteria (in 0.9% NaCl; the turbidity of the suspension was equal to a McFarland 0.5 standard) were streaked with a swab to cover the entire surface of an L agar plate. After the surface had dried, the E Test strip was placed on it and the plate was incubated for 18 h at 37°C. The MIC was then interpreted by noting the point of intersection of the growth margin with the MIC scale of the E Test strip.

Susceptibility determinations by the agar diffusion method were performed by using L agar plates and antibiotic discs as previously described (17). The antibiotic discs (A/S Rosco, Copenhagen, Denmark) contained mupirocin (10 μg), vancomycin (70 μg), bacitracin (40 units), and rifampin (30 μg).

Measurement of enzyme activities. Detection of β-lactamase was done by the modified method of O'Callaghan et al. (32). Nitrocefin (Glaxo Pharmaceuticals, Ltd., Greenford, England) was added to the enzyme samples in 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) (pH, 7.2) to yield a final concentration of 100 μg/ml, and the rate of nitrocefin hydrolysis was then monitored at 540 nm. The activity of glucose 6-phosphate dehydrogenase was determined as described by Langdon (23). Cellular enzyme activities were determined from cells sonicated twice for 15 s each time at an amplitude of 12 μm under the sonication microtip of a Soniprep 150 (Measuring & Scientific Equipment, Ltd., London, England).

RESULTS

Antibiotic-susceptible phenotype of the *omsA* mutant. We first verified and extended the finding of Tsuruoka et al. (39) that *omsA* mutant strain CDH23-213 is remarkably susceptible to certain antibiotics. The MICs of rifampin and fusidic acid were approximately 250-fold lower for the *omsA* mutant strain than for its isogenic *omsA*$^+$ sister transductant (CDH23-210), and those of erythromycin, mupirocin, and clindamycin were 128-, 64-, and 16-fold lower, respectively (Table 2). The *omsA* mutant strain was also susceptible to bacitracin and vancomycin (Table 2). Common to all of these antibiotics is the fact that they do not penetrate the enterobacterial OM through the water-filled, narrow porin chan-

TABLE 2. Complementation of the *omsA* mutant phenotype as determined by measurement of MICs of various antibiotics[a]

Antibiotic	MIC (μg/ml) for:			
	CDH23-210 (*omsA*$^+$)	CDH23-213 (*omsA*)	CDH23-213/ pUCHS14-II (*omsA ssc*$^+$)	CDH23-213/ pUCHS16A (*omsA ompH*$^+$)
Fusidic acid	512	2	512	2
Rifampin	4	0.015	2	0.015
Clindamycin	128	8	128	8
Bacitracin	≥1,024	32	≥1,024	32
Erythromycin	32	0.25	64	0.5
Vancomycin	128	32	128	32
Mupirocin	64	1	64	0.5

[a] MICs were determined at 28°C in L broth (supplemented with ampicillin when appropriate).

nels, since they are either hydrophobic molecules (rifampin, fusidic acid, erythromycin, mupirocin, and clindamycin) or large, hydrophilic molecules (bacitracin and vancomycin) (28, 30, 37, 40). In contrast, no sensitization or very slight sensitization to antibiotics such as cefuroxime and benzylpenicillin, which use the porin channels, was observed (data not shown; see also reference 39). Furthermore, like other enteric bacteria which have a defective or damaged OM (28, 37, 40), the *omsA* strain showed no increased susceptibility to aminoglycoside antibiotics (data not shown; see also reference 39).

***omsA* mutant phenotype is complemented by *ssc*.** As detailed in the introduction, both the gene mapping data and comparison of the phenotypic properties prompted us to search for a possible relationship between *omsA* and *firA*. Since we had previously cloned and sequenced the *firA* analog of *S. typhimurium*, the *ssc* gene (16), we tested whether it could complement the *omsA* mutation. We employed pUC19-derived multicopy plasmid pUCHS14-II (17), which carries *ssc*$^+$ as its only inserted gene, and introduced it into CDH23-213. The transformant was as resistant to all of the antibiotic probes tested as was the *omsA*$^+$ control (Table 2). Also, the ability to grow at 42°C was resumed. Accordingly, *ssc* fully complemented the *omsA* mutant phenotype. Another pUC19-derived plasmid, pUCHS16A (17), which lacks *ssc* but contains the 5′-flanking gene *ompH*, did not elicit any complementation (Table 2).

Complementation with different *ssc* alleles. To study further the ability of the *ssc* gene to reverse the *E. coli omsA* mutation, we tested various *ssc* mutant alleles for this property. Plasmids pSSC101 to pSSC112 (Table 1) contain 12 *ssc* alleles previously made by site-directed mutagenesis by us (19). pSSC101 corresponds to the *ssc-1* mutant allele (originally found in *S. typhimurium* mutant strain SH7622), and the rest of the alleles encode other mutant Ssc proteins, each having a single amino acid alteration. The ability of these plasmids to complement the *omsA* mutation of *E. coli* (Table 3) appeared to be very similar to their ability to complement the *ssc* mutation of *S. typhimurium* (19). Accordingly, pSSC101, pSSC109, pSSC110, and pSSC111, which encode mutant Ssc proteins with substitutions of Val-291→Met, Met-288→Lys, Met-290→Lys, and Met-292→Lys, respectively, caused only partial complementation. The very defective *ssc* allele (Gly-289→Asp; carried by pSSC112), which potentiates even the antibiotic-sensitive phenotype of SH7622 (19), was completely unable to reverse the *omsA* phenotype. The *ssc* allele in pSSC102 (Met-288→Leu) made the *omsA* strain fully resistant (Table 3) but made the *ssc-1* mutant only partially resistant (19). The remaining six pSSC plasmids made the *omsA* mutant nondefective, exactly as they changed the *ssc1* mutant. Put together, all of these results further support our idea that *omsA* is allelic to *firA*, the *E. coli* analog of the *ssc* gene of *S. typhimurium*.

Cloning and sequencing of the *omsA* allele of *firA*. We then cloned and sequenced the *firA* gene (i.e., the *omsA* allele) of

TABLE 3. Ability of plasmid-encoded *ssc* and *firA* alleles to complement the *omsA* mutation in CDH23-213

Strain/plasmid	*ssc* or *firA* allele in plasmid	Sensitivity[a] to:				Sensitivity interpretation[b]
		Mupirocin	Vancomycin	Bacitracin	Rifampin	
CDH23-213	None	13 (24)	11 (18)	17 (27)	29 (20)	SS (SS)
CDH23-213/pUCHS14-II	*ssc*$^+$	0	2	0	14	R
CDH23-213/pSSC101	*ssc-101*	3	9	10	21	S
CDH23-213/pSSC102	*ssc-102*	0	4	0	16	R
CDH23-213/pSSC103	*ssc-103*	0	4	0	16	R
CDH23-213/pSSC104	*ssc-104*	0	4	0	15	R
CDH23-213/pSSC105	*ssc-105*	0	4	0	16	R
CDH23-213/pSSC106	*ssc-106*	0	3	0	15	R
CDH23-213/pSSC107	*ssc-107*	0	3	0	14	R
CDH23-213/pSSC108	*ssc-108*	0	2	0	14	R
CDH23-213/pSSC109	*ssc-109*	2	9	8	20	S
CDH23-213/pSSC110	*ssc-110*	0	10	11	21	S
CDH23-213/pSSC111	*ssc-111*	0	10	10	20	S
CDH23-213/pSSC112	*ssc-112*	18	13	20	35	SS
CDH23-213/pOMSA101	*omsA*	0 (6)	2 (4)	0 (4)	12 (12)	R (S)
CDH23-213/pOMSA102	*omsA*[c]	0 (4)	2 (3)	0 (3)	13 (10)	R (S)
CDH23-213/pOMSA103	*firA*$^+$	0 (0)	1 (1)	0 (0)	11 (7)	R (R)
CDH23-213/pGAH317	*firA*$^+$	0	1	0	13	R
CDH23-210 (*omsA*$^+$ control)	None	0 (0)	1 (0)	0 (0)	13 (8)	R (R)

[a] Sensitivity to antibiotics was measured as inhibition zones (in millimeters) around antibiotic discs. Determinations were done on L agar plates (with ampicillin [100 μg/ml] when plasmids were used) at 28°C. In parentheses are the corresponding values obtained at 39°C. The diameter of the antibiotic disc (9 mm) is subtracted.

[b] R, resistant; S, sensitive; SS, very sensitive. Interpretations in parentheses are based on sensitivity determinations performed at 39°C.

[c] Plasmid pOMSA102 contains the *firA* mutant allele *omsA* made by site-directed mutagenesis.

TABLE 4. Complementation of the antibiotic-sensitive phenotype of CDH23-213 by plasmids containing the *omsA* allele as determined at 37°C[a]

Strain/plasmid	*firA* allele in plasmid	MIC (μg/ml) of:			
		Vancomycin	Fusidic acid	Rifampin	Erythromycin
CDH23-213	None	8	2	0.016	0.125
CDH23-213/pOMSA101	*omsA*	32	64	1.5	3
CDH23-213/pOMSA102	*omsA*[b]	32	48	1	2
CDH23-213/pOMSA103	*firA*$^+$	>256	>256	6	12
CDH23-210	None	>256	>256	8	32

[a] MICs were determined by using the E Test (see Materials and Methods) in L agar plates (with ampicillin [100 mg/ml] when plasmid-containing strains were studied).
[b] Prepared by site-directed mutagenesis.

CDH23-213. The *firA*-containing chromosomal 1.4-kb *Pst*I fragment was ligated to pUC19 and identified by Southern hybridization. Also, the wild-type *firA* gene (from plasmid pGAH317) was cloned to pUC19. The recombinant plasmids were then tested for the ability to complement the *omsA* mutation of CDH23-213. If the *firA* gene was proximal to *lac* and in the opposite orientation, not even plasmids with *firA*$^+$ elicited any complementation (data not shown). On the other hand, if the wild-type *firA* gene was in the same orientation as the *lac* promoter and distal to it (pOMSA103), it fully complemented the *omsA* phenotype (Table 3). The identical construction containing the *omsA* allele (pOMSA101) also complemented the phenotype. However, when the complementation tests were performed at 39°C (instead of 28°C), pOMSA101 proved to complement more weakly than pOMSA103 (Table 3). Consistent with the difference observed in Table 3, the MICs of vancomycin, erythromycin, rifampin, and fusidic acid at 37°C were four- to eightfold lower when CDH23-213 carried pOMSA101 than when it carried pOMSA103 (Table 4).

The *omsA* allele was sequenced by using oligonucleotide primers. Compared with the wild-type *firA* sequence (5), the sequence was identical except for one point mutation. There was a G-to-A transition in nucleotide 812 (the numbering is consistent with Fig. 4 of reference 5). This changed codon 271 from serine to asparagine. The same mutation was also made by site-directed mutagenesis. This *omsA* allele behaved the same way as the original *omsA* allele in the complementation assays (Tables 3 and 4).

The thermosensitive phenotype of the *ssc-1* mutant compared with that of the *omsA* mutant. As *omsA* and *ssc-1* mutations are allelic, we compared the thermosensitive phenotypes caused by these two mutations. After the temperature was shifted from 28 to 42°C, the growth of both of the mutants stopped after 1.5 h (Fig. 1). The CFU/milliliter fell 1 to 2 log units in SH7622 and nearly 3 log units in CDH23-213.

One distinct property of the *omsA* mutant is that it releases periplasmic proteins upon a temperature shift up (39). To study whether this too is a characteristic of the *ssc-1* mutant, we introduced plasmid vector pUC19 (which encodes β-lactamase) into SH7622. The SH7622 derivative which carries pUC19-derived pUCHS14-II (which encodes both β-lactamase and wild-type Ssc) was used as a control. As shown in Fig. 2, a temperature shift from 28 to 42°C (but not to 37°C) did induce marked leakage of the periplasmic enzyme from SH7622/pUC19. The activity in the medium as a percentage of the total activity was 82% at 42°C and 4% at 37°C (the values with the control plasmid were 2 and 0.3%, respectively). Accordingly, the OM of SH7622 became functionally very defective at 42°C and allowed leakage of the periplasmic contents. In contrast, protein leakage through the cytoplasmic membrane was minimal even at 42°C; less

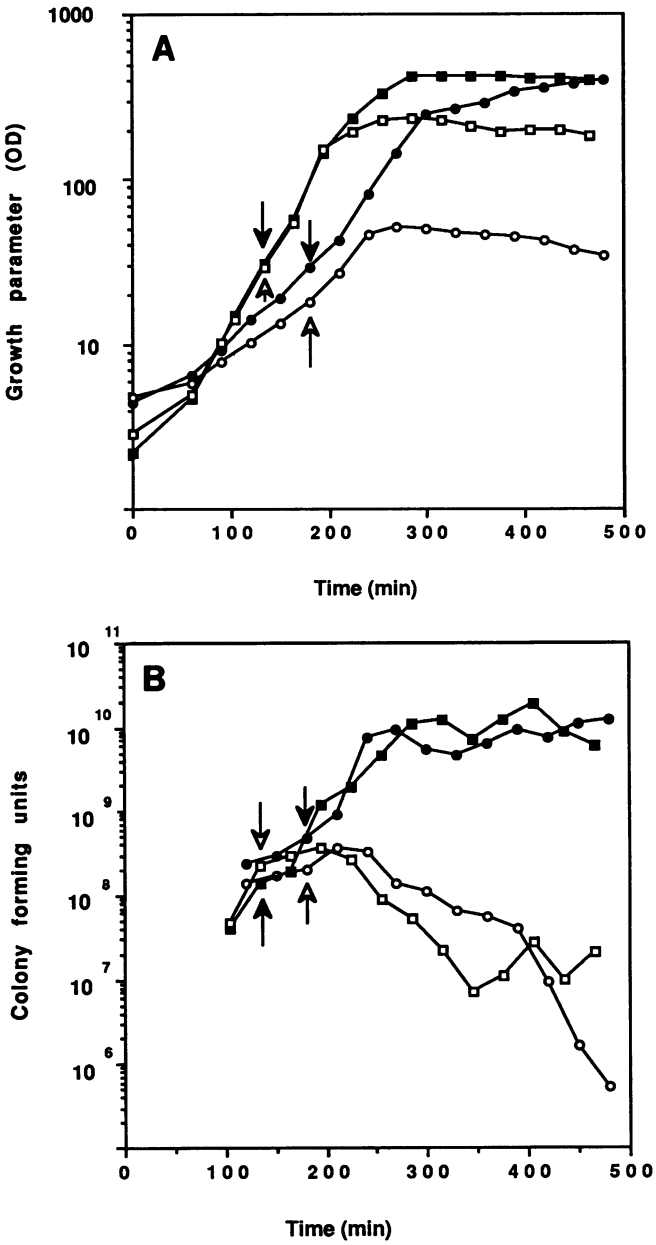

FIG. 1. Thermosensitivity of growth as determined for two strain pairs with analogous mutant alleles. CDH23-213 (*omsA*) and its isogenic wild-type control, CDH23-210, and SH7622 (*ssc-1*) and its wild-type parent, SH5014, were used. Growth was measured as A_{550} (A), and viability was determined as CFU/milliliter (B). The cultures were incubated at 28°C until they reached an optical density (OD) of approximately 0.2 to 0.3 (arrows), and then the temperature was shifted to 42°C. Symbols: ●, CDH23-210; ○, CDH23-213; ■, SH5014; □, SH7622.

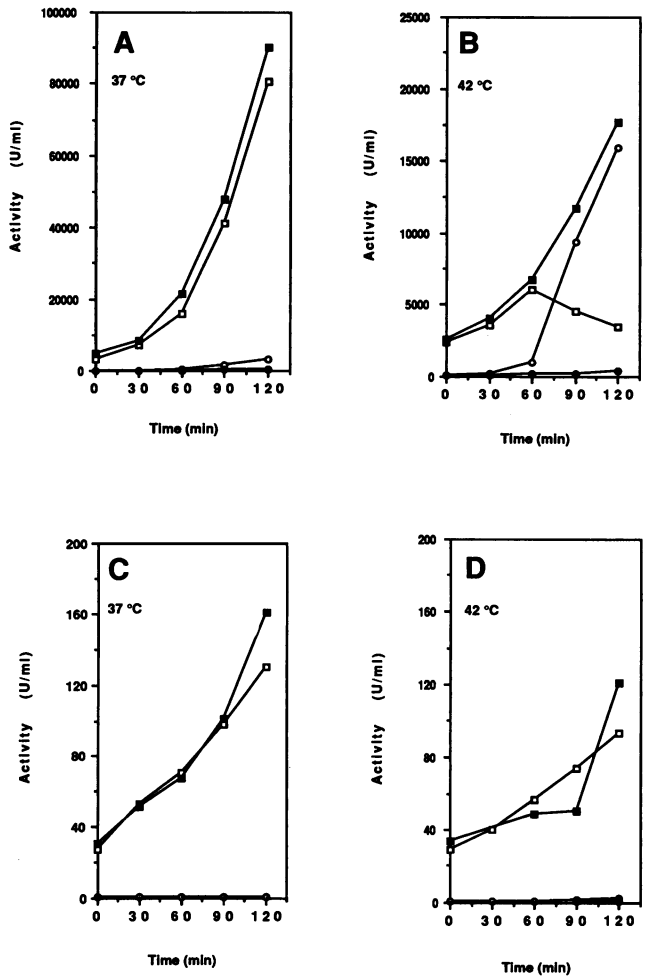

FIG. 2. Release of periplasmic *amp*-encoded β-lactamase and cytoplasmic glucose 6-phosphate dehydrogenase from *ssc-1* mutant strain SH7622. SH7622 carrying either pUC19 (*amp*, open symbols) or pUCHS14-II (*amp ssc*$^+$, closed symbols) was shifted from 28 to 37°C (A and C) or to 42°C (B and D) at time zero. β-Lactamase (A and B) and glucose 6-phosphate dehydrogenase (C and D) activities in the growth medium (○, ●) and the corresponding cellular activities (□, ■) are shown. Units are nanomoles per minute.

than 3% of the total activity of the cytoplasmic marker enzyme (glucose 6-phosphate dehydrogenase) was found in the medium after 2 h at 42°C (Fig. 2).

DISCUSSION

The *firA* mutant (*firA200*) of *E. coli* was first described in 1970 (24). It was a rifampin-sensitive phenotypic revertant (hence, *fir* versus *rif*) of the *E. coli rpoB* mutant strain, which carries the rifampin-resistant β subunit of RNA polymerase. The MIC of rifampin for the *firA rpoB* strain was approximately threefold lower than the MIC for the *rpoB* strain (25). Furthermore, the *firA rpoB* strain was thermosensitive and its RNA synthesis halted at 42°C (25). On the basis of this evidence, the *firA* mutation was believed to affect RNA transcription. Later, Dicker and Seetharam (5) constructed strains that overproduce the product of *firA*, the 36-kDa FirA protein, and showed that an antiserum to RNA polymerase holoenzyme coimmunoprecipitates FirA from such strains. They concluded that FirA has a physical association with the transcriptional machinery and is directly involved in transcription (5). We, however, linked *firA* to an apparently very different cellular function by finding that a point mutation in the *ssc* gene, the *firA* analog of *S. typhimurium*, results in both thermosensitivity and, at the permissive temperature, a drastic increase in susceptibility to a large set of hydrophobic antibiotics, dyes, and detergents (17). Since this unselective increase in susceptibility to hydrophobic agents indicates a defect in the OM permeability barrier to such agents, we suggested that the *firA* gene is involved in generation of the OM.

The present investigation studied the conditionally lethal *omsA* mutant of *E. coli*, which has been shown by Tsuruoka et al. (39) to have a very defective OM permeability barrier function at growth-permissive temperatures. We also showed that this mutation was due to a base pair substitution in *firA*, i.e., the gene mutated in the above-mentioned phenotypically similar *ssc-1* mutant of *S. typhimurium*. Furthermore, the results revealed that the OM of the *firA* mutants becomes ruptured at nonpermissive temperatures, as indicated by leakage of the periplasmic β-lactamase into the medium. Even more interestingly, we were able to show that this rupturing took place under conditions in which the cytoplasmic membrane remained impermeable to cytoplasmic enzymes. These results further strengthen our concept that the *firA* gene has an essential function in OM biosynthesis. Furthermore, the results also strongly suggest that this gene functions identically in *E. coli* and *S. typhimurium*, as proposed previously by us (17).

The *firA* genes of *S. typhimurium* (16) and *E. coli* (5) are 88% identical. The corresponding proteins have a molecular mass of 36 kDa and are 96% identical. Downstream of *firA*, 560 bp apart, lie *lpxA* (3) and then *lpxB* (3, 4). These are the only known genes of lipid A biosynthesis and might be cotranscribed with *firA* (see the references in reference 6). When *firA* of *S. typhimurium* was compared with all of the bacterial genes in the GenBank library, the best sequence homology was found with *lpxA* (47). Consistently, among all of the SWISS-PROT protein sequences, the best match with FirA was *E. coli* UDP-acetylglucosamine *O*-acyltransferase (the LpxA protein) (47). The FirA and LpxA proteins share two long regions of distinct homology (homology allowing equivalent amino acids, 43%) and a peculiar hexapeptide repeat theme also found in three acetyltransferases (6, 41, 47). This homology with LpxA suggests that FirA is involved in lipid A biosynthesis and that it has a function which resembles that of LpxA, i.e., an acyltransferase function. At least six to eight of the enzymes directly involved in lipid A synthesis await molecular characterization (33). These include UDP-3-*O*-(3-hydroxymyristoyl)-acetylglucosamine deacetylase and UDP-3-*O*-(3-hydroxymyristoyl)-glucosamine *N*-acyltransferase (which catalyze the second and third steps of synthesis, respectively), lipid A 4′ kinase, and the late acyltransferases (i.e., lauroyl and myristoyl transferases). Work is in progress in our laboratory to elucidate the possible functional role of FirA in the biogenesis of lipid A. Indirect evidence favoring such a role comes from our finding that the rate of lipid A synthesis, relative to that of phospholipid synthesis, is drastically decreased in the *firA* mutant at 42°C (13). Furthermore, the mature LPS molecules produced by the *firA* mutant at 42°C are modified to contain palmitic acid (12, 13).

The *lpxA2* mutant of *E. coli* isolated and characterized by Galloway and Raetz is the best-characterized mutant strain defective in lipid A biosynthesis (7). It synthesizes drasti-

cally reduced amounts of lipid A at 42°C and rapidly dies at that temperature. We have recently shown (48) that this strain is extremely susceptible to hydrophobic antibiotics (including rifampin, fusidic acid, erythromycin, and clindamycin) at growth-permitting temperatures. Their MICs are 32- to >128-fold lower for this strain than for the parental type strain, while the MICs of such antibiotics which use the porin channels through the OM are almost unchanged. The very low MIC values of the hydrophobic antibiotics determined for the *lpxA2* strain are reminiscent of those determined for the *omsA* mutant in the present study. The *lpxA2* and *omsA* mutants are the most antibiotic-supersusceptible enterobacterial strains ever published. Accordingly, the *lpxA* and *firA* mutants are phenotypically very similar.

The 5'-flanking region of *firA* is occupied by *hlpA* (also known as *ompH*), which encodes cationic 17-kDa protein HLP-I (histone-like protein I, the OmpH protein). This protein has a very conservative structure among the members of the family *Enterobacteriaceae* (15, 18, 20, 22, 43, 46), carries a signal sequence that is characteristic of secreted proteins in its precursor form (18, 20, 21, 43), can be found in isolated OM (18, 22), and has an unknown function. Only three nucleotides separate the *hlpA* stop and *firA* initiation codons (5, 16). We have previously shown by using minicells and by complementation assays that the *firA* gene of *S. typhimurium* is expressed in the absence of the *hlpA* promoter, i.e., in a plasmid which lacks the 5' terminus of *hlpA* and where the vector-derived *lac* promoter is opposite in orientation to *firA* (17). In the present report, we show that this construction is also able to complement the *omsA* mutant allele, while an analogous construction containing the *firA* gene of *E. coli* is inactive. Only those *firA* inserts of *E. coli* in which *firA* is under the *lac* promoter have complementing activity. We have previously suggested that *firA* of *S. typhimurium* might use a promoter of its own and have identified a putative promoter region 159 nucleotides upstream that overlaps the protein-encoding region of *hlpA* (16). In *E. coli*, this region is less homologous to the canonical promoter sequence. Accordingly, it could be suggested that *firA* is normally cotranscribed with *hlpA* but that the *firA* gene of *S. typhimurium* can also utilize, to some extent, the additional promoter mentioned above.

When present in the multicopy plasmid under the *lac* promoter, the *omsA* mutant allele is able to partially complement the phenotype of the chromosomal *omsA* mutant. This is probably due to overexpression of the plasmid-borne gene in such a construction. An analogous construction containing the *firA200* mutant allele (see below) complements the rifampin-sensitive phenotype of the *firA200* mutant (Table 4 of reference 5). Apparently, no such overexpression takes place in plasmids in which *S. typhimurium firA* is not under *lac* control and uses its own additional promoter (as suggested above), since, as shown in this report, the defective *firA* alleles of *S. typhimurium* (*ssc-1* and the defective missense base pair substitution mutants made by site-directed mutagenesis) complement the *omsA* phenotype only weakly or do not complement it at all (whereas the wild-type *firA* of *S. typhimurium* and the nondefective in vitro mutant alleles complement it fully).

The mutant FirA protein described in this report has only one amino acid change, Ser-271→Asn. Also, the FirA protein encoded by the *ssc-1* allele has a single alteration, Val-291→Met (17). Accordingly, both the thermosensitive and antibiotic-supersusceptible phenotypes are conferred by a single mutation. On the other hand, the rifampin-sensitive and thermosensitive phenotype-causing mutation *firA200* (see above) encodes a protein with a total of three different amino acid alterations, Gln-165→Arg, Gly-228→Asp, and Gly-252→Ser (5). Its spontaneous mutation, *firA201*, is not rifampin sensitive and encodes a protein which has preserved the Gly-228→Asp and Gly-252→Ser mutations and lacks the Gln-165→Arg mutation but has Ile-172→Phe instead (5). On the basis of preliminary transcomplementation studies, Dicker and Seetharam have suggested that Gly-228→Asp and Gly-252→Ser are responsible for the thermosensitive and rifampin-sensitive phenotypes of the *firA200* mutant (6). They have noted also that both of these mutations are located in turn residues (i.e., residue *b*, *c*, or *d* of the imperfect hexapeptide tandem repeat units) that favor formation of the hypothetical β-turn structure of the protein (6). However, neither Ser-271→Asn nor Val-291→Met is located in such residues. Regarding the 11 novel FirA proteins made by site-directed mutagenesis of *firA*, those with Met-288→Lys and Gly-289→Asp are the most defective ones in complementation assays, followed by the proteins with Met-290→Lys and Met-292→Lys. The plasmid that encodes Gly-289→Asp appears to sensitize the *ssc-1* and *omsA* mutants further (reference 19 and this study) but has only a negligible effect on the sensitivity of the *firA*$^+$ strain (19). This can be taken to suggest that this mutant protein is even more defective than the proteins with Val-291→Met and Ser-271→Asn and that it competes with them for binding to the hypothetical ligand. Also, the Gly-289→Asp mutation does not map in any of the hypothetical turn residues. There are no empirical data on the tertiary structure of the FirA protein. In the absence of any crystallographic data, it is very difficult to draw any conclusions regarding the effect of the identified amino acid alterations on the conformation of the protein.

The various *firA* mutant strains can certainly be expected to differ in the extents of their functional lesions. The *firA201* mutant apparently carries a less defective allele than the *firA200* mutant, since it is not supersusceptible to rifampin under conditions in which the *firA200* strain is (5, 6). Among the phenotypically characterized mutants, the *omsA* mutant is the most defective. Even though the phenotypes of the *omsA* and *ssc-1* mutants are very similar, some differences can be found. On the basis of the MIC values (reference 17 and this study), we conclude that the *omsA* mutant is even more susceptible to all of the tested antibiotics than is the *ssc-1* mutant. Furthermore, it leaks its periplasmic enzymes at 37°C (39), whereas in the *ssc-1* mutant no leakage was detected at that temperature (this study). Moreover, the *omsA* mutant releases part of its cytoplasmic constituents also and starts to lyse (39), whereas the *ssc-1* strain releases exclusively its periplasmic material. In the elucidation of the function of FirA, the most defective *firA* mutant (i.e., the *omsA* strain) could be expected to be the most informative one.

Since the *firA* and *lpxA2* mutants are extremely supersusceptible to hydrophobic agents, we have previously suggested (19, 48) that they could be useful tools in studies of bacterial physiology, as well as in applied bacteriology and biotechnology, all fields in which the permeability barrier properties of the OM potentially pose problems. Furthermore, phenotypic revertants of both types of mutants could help us to evaluate whether lipid A is an essential molecule. The only evidence for the assumption that lipid A and its attached ketodeoxyoctulosonates are essential for the viability of the bacteria comes from the findings that the *lpxA2* mutants and the *kds* mutants (defective in ketodeoxyoctulosonate synthesis) are conditionally lethal and that inhibi-

tors of ketodeoxyoctulosonate synthesis are antibacterial (7, 9–11, 31, 33). Whether bacteria can live without lipid A if suitable bypass mutations are also present remains to be seen. Since the OM is certainly essential for pathogenic bacteria (44), inhibitors of lipid A biosynthesis could be a very promising new class of antibacterial agents. Molecular characterization of all of the enzymes involved in lipid A biosynthesis would greatly advance the development of such agents.

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