The lipid A biosynthesis deficiency of the *Escherichia coli* antibiotic-supersensitive mutant LH530 is suppressed by a novel locus, ORF195

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A new mutant of *Escherichia coli* K-12 supersensitive to both hydrophobic and large hydrophilic antibiotics was isolated and characterized. The mutant grew well at 28 °C, poorly at 37 °C, and did not grow at 42 °C. The rate of its lipid A biosynthesis was reduced as compared to that of the parent strain. This deficiency was rescued by a novel locus, ORF195, the function of which has not been elucidated. ORF195 is located in the 76 min region in the *E. coli* chromosome and encodes a hypothetical 21·8 kDa protein with no signal sequence. ORF195 isolated from the mutant strain had an identical sequence to the wild-type allele, indicating a suppressor function of the gene product.

Keywords: *Escherichia coli*, ORF195, lipid A, antibiotic-supersensitive mutant, permeability mutant

INTRODUCTION

The outer membrane (OM) of Gram-negative enteric bacteria functions as an effective permeability barrier (for reviews, see Nikaido & Vaara, 1985, 1987). Hydrophobic and large hydrophilic compounds are effectively excluded due to lipopolysaccharide (LPS) molecules that are joined electrostatically by Mg²⁺, Ca²⁺ and polyamines to form a rigid and stable outer leaflet (Nikaido & Vaara, 1987; Nikaido, 1990; Koski & Vaara, 1991). Although the structure of the OM of *Escherichia coli* and *Salmonella typhimurium* is quite well known, its biogenesis is poorly understood as are many structure-function relationships. Furthermore, the biosynthesis of lipid A, the essential component of LPS, has not been fully elucidated (Raetz, 1990, 1993; Schnaitman & Klena, 1993; Young *et al.*, 1993; Clementz *et al.*, 1995), and the regulation of the biosynthesis pathway and the final steps of assembly and translocation of LPS are unclear.

Mutants supersensitive to antibacterial agents have been useful in characterization of the barrier function of OM. Heptose-deficient deep-core LPS mutants of *S.*

typhimurium and *E. coli* are the most studied (for reviews, see Nikaido & Nakae, 1979; Nikaido & Vaara, 1985; Vaara, 1993). Many supersusceptible mutants harbour defects in lipid A biosynthesis genes. Such mutants include the *lpxA* and *lpxD* mutants of *S. typhimurium* and *E. coli* (Sukupolvi *et al.*, 1984; Hirvas *et al.*, 1991a; Vuorio & Vaara, 1992a, b; Vaara, 1993). Some other supersusceptible mutants, such as the *acrA* mutant of *E. coli*, have recently been shown to be defective in efflux pump systems (Ma *et al.*, 1995). The biochemical defects of many other antibiotic-susceptible mutants, such as the SSB and *lkyD* mutants of *S. typhimurium* and the *abs* mutant of *E. coli*, have not yet been elucidated.

As antibiotic-susceptible mutants have been shown to be valuable in studies of OM biogenesis, we isolated a new set of supersensitive *E. coli* mutants. Here we describe one of them, *E. coli* LH530. The mutant is supersensitive to several unrelated hydrophobic antibiotics and does not grow at high temperatures. We also show that it synthesizes remarkably reduced amounts of lipid A at higher temperatures. Furthermore, we report that these defects are suppressed by multiple copies of a previously uncharacterized gene, ORF195, located at 76 min on the *E. coli* chromosome. However, ORF195 isolated from the mutant strain has an identical sequence to the wild-

Abbreviations: OM, outer membrane.

type allele. This indicates that ORF195 has a suppressor function.

METHODS

Strains, plasmids and growth conditions. *E. coli* K-12 strain JM105 (Yanisch-Perron *et al.*, 1985) was used as the parent strain for mutant isolation and also for the preparation of an *E. coli* gene bank. *E. coli* strain DH5 α (Hanahan, 1983) was used as the cloning host. The cloning vector was pUC19 (Pharmacia). The low-copy vector was pACYC184 (New England Biolabs).

The properties of the OM mutant strains SM101 (*lpxA*; Galloway & Raetz, 1990), CDH23-213 (*lpxD*; Tsuruoka *et al.*, 1988) and D21f2 (*rfa*, LPS chemotype Re; Boman & Monner, 1975), and their parents SM105 (*lpxA*⁺), CDH23-210 (*lpxD*⁺), and D21 (*rfa*⁺) have recently been reviewed (Vaara, 1993).

Bacteria were grown in LB broth at 28 °C, 37 °C or 42 °C. When necessary, antibiotics were added at the following concentrations: 100 μ g ampicillin ml⁻¹, 40 μ g tetracycline ml⁻¹, 40 μ g chloramphenicol ml⁻¹ and 30 μ g fusidic acid ml⁻¹.

Mutagenesis and isolation of supersensitive mutants. Several cultures of JM105 grown overnight at 37 °C were incubated with 0.5% diethyl sulfate for 30 min at 37 °C. Cultures were diluted 1/100 and incubated overnight at 28 °C. Mutants supersensitive to a hydrophobic antibiotic, fusidic acid, were isolated by penicillin counterselection (Tamaki *et al.*, 1971; Coleman & Leive, 1979; Sukupolvi *et al.*, 1984). Approximately 10⁸ cells ml⁻¹ were grown overnight at 28 °C in the presence of penicillin (300 μ g ml⁻¹), and fusidic acid (30 μ g ml⁻¹). Survivors were plated on LB plates. After growth at 28 °C the plates were replica-printed onto three plates. One of the plates contained fusidic acid (30 μ g ml⁻¹) and was incubated at 28 °C. The other two plates lacked fusidic acid and were incubated at 28 °C and 42 °C, respectively. Clones unable to grow in the presence of fusidic acid and at 42 °C were picked for further studies.

Antibiotic susceptibility determinations and phage sensitivity testing. Antibiotic susceptibility determinations by the agar diffusion method (Hirvas *et al.*, 1991a) were performed by using LB plates and antibiotic discs. The minimal inhibitory concentrations (MICs) of the antibiotics (E test; AB Biodisk) were measured as described previously (Vuorio & Vaara, 1992a). Bacterial sensitivity to LPS-specific bacteriophages C21, Br2, Br60 and Ffm was determined by the drop-on-lawn method on LB plates (Wilkinson *et al.*, 1972).

DNA manipulations. General DNA manipulations were carried out as described by Sambrook *et al.* (1989). Isolation of chromosomal DNA was done as previously described (Koski *et al.*, 1989). Plasmid DNA was purified on Qiagen spin columns according to the manufacturer's instructions. Southern blotting was performed under alkaline conditions with positively charged nylon membranes (Boehringer Mannheim). Southern hybridization was done at 37 °C in 5 $\times$ SSC, 0.5% sodium dodecyl sulfate (SDS), 5 $\times$ Denhardt's solution, 50% (v/v) formamide. The hybridized filter was washed at room temperature in 4 $\times$ SSC, 0.5% SDS for 1 h. Autoradiography was carried out at room temperature for 2–12 h. The 900 bp *Pst*I-*Eco*RV fragment originating from plasmid pLH27 (see Fig. 2) was labelled by using a multiprime DNA labelling kit (Amersham) and used as a probe. Appropriate restriction fragments were cut out of the agarose gel and DNA was isolated as previously described (Hirvas *et al.*, 1991b). DNA sequencing of double-stranded DNA cloned in

pUC19 was performed by robotic work station catalyst 800 (Applied Biosystems) using fluorescently labelled primers or fluorescently labelled dye terminators with respective sequencing kits supplied by Applied Biosystems. Reactions were analysed using an Applied Biosystems 373 Stretch Automatic Sequencer.

Construction of a chromosomal clone bank. Chromosomal DNA was prepared from *E. coli* JM105, partially digested with *Sau*3A [approximately 0.12 U (μ g DNA)⁻¹, 37 °C, 1 h]. Fragments 5–10 kb in size were isolated and ligated into *Bam*HI-digested pUC19 and treated with alkaline phosphatase. The ligated mixture was used to transform *E. coli* DH5 α . Transformants were selected on LB plates containing ampicillin. Approximately 8000 transformants were pooled and plasmid DNA was extracted from this pool.

Isolation of complementing clones from the gene bank. Part of the plasmid DNA of the bank was used to transform competent cells of the supersusceptible mutant LH530. The transformation mixture was plated onto LB plates containing fusidic acid (30 μ g ml⁻¹). Three clones which were repeatedly able to grow on fusidic-acid-containing plates were found. To confirm that these clones were not chromosomal revertants of LH530, plasmid DNA was isolated from the clones and once again introduced into LH530.

Isolation of ORF195 from *E. coli* LH530. Chromosomal DNA isolated from strain LH530 was digested with *Bgl*I; end-filling by Klenow was followed by *Sph*I digestion and the fragments were run on 0.8% agarose gels. Fragments approximately 1.8–2.2 kb in size were isolated and ligated to *Sph*I- and *Hinc*II-digested pUC19. The hybridization probe described above was used to detect the clone that carried ORF195. This plasmid was named pLH32.

Northern blot analysis. Total RNA was isolated from exponentially growing (37 °C) cells by the hot phenol method (VonGabain *et al.*, 1983). The RNA was size-fractionated in formaldehyde gel containing 1.2% (w/v) agarose (Sambrook *et al.*, 1989) and transferred to zeta-probe membranes under mild alkaline conditions (50 mM NaOH). The blots were hybridized with the ORF195-specific probe. The *Eco*RV-*Hpa*I fragment of 300 bp that is completely internal to the ORF195 gene was labelled with [α -³²P]CTP using a random priming kit from Stratagene. The hybridization was done at 42 °C in 5 $\times$ SSC, 0.5% SDS, 5 $\times$ Denhardt's solution, 50 mM Na₂HPO₄ (pH 7.5), 50% formamide. The hybridized filter was washed in 2 $\times$ SSC, 0.1% SDS for 1 h and in 0.5% SSC, 0.1% SDS for 0.5 h at 42 °C. The blot was imaged in a Bio-imaging analyser (Bas-1500, Fuji) and bound [α -³²P]CTP was analysed and quantified by using the MacBas v. 2.x program (Fuji Photo Film).

Determination of the ratio of LPS to phospholipid biosynthesis. The ratio of LPS and phospholipid biosynthesis in LH530 and its derivatives was assayed (Vuorio & Vaara, 1995) after labelling their fatty acids with radioactive acetate *in vivo* at 28 °C, 37 °C and 42 °C. In detail, 5 $\times$ 10⁸ bacterial cells (from fresh LB plates grown at 28 °C) were suspended in 5 ml LB broth. After growing (with stirring) for 1 h at the selected temperature (28 °C, 37 °C or 42 °C), 10 μ l (0.74 MBq) [¹⁴C]acetate (sodium salt, CFA229, specific activity 1.89 GBq mmol⁻¹; Amersham) was added and growth was continued for an additional 2 h. The cells were harvested by centrifugation (4300 r.p.m., Heraeus megafuge), washed with 2.5 ml saline, transferred with 1 ml of saline into Sarstedt tubes (1 ml) and collected by centrifugation. The phospholipids were extracted with two consecutive volumes of 750 μ l chloroform/methanol (1:2). The radioactivity of the extract was determined by measuring that of a 75 μ l sample in a liquid

scintillation counter. From the remaining cell debris the ester-linked fatty acids of LPS were liberated by hydrolysis with 300 µl 0.17 M NaOH at 100 °C for 1 h. Thirty microlitres of 0.5 M sodium acetate (pH 5.0) was added, the mixture was neutralized by adding 30 µl 1.7 M HCl and converted to a two-phase system by addition of 800 µl chloroform/methanol (1:1). After low-speed centrifugation the lower phase was concentrated, dissolved in 50 µl chloroform/methanol (1:2) and its radioactivity was measured. The result was multiplied by a factor of 1.5, as one third of the fatty acids of LPS, the amide-linked ones, resist NaOH hydrolysis. The ratio of LPS to phospholipid biosynthesis was calculated by dividing the radioactivity of the LPS fraction (multiplied by 1.5) by the radioactivity of the phospholipid fraction.

Qualitative analysis of the lipid A by TLC. Bacteria grown in the presence of radioactive acetate as described above were subjected to extraction with chloroform/methanol (1:2). LPS residing in the pellet was hydrolysed to obtain lipid A 4'-monophosphate with 0.5 ml 0.1 M HCl for 1 h at 100 °C (Galloway & Raetz, 1990), pelleted by centrifugation for 10 min at 15000 g, dried and dissolved in 200 µl methanol. Samples of this preparation were analysed by TLC using silica gel 60 plates (Merck) and chloroform/methanol/water/25% (v/v) NH₄OH (40:25:4:2, by vol.) as solvent. The radioactive bands were visualized by autoradiography employing Kodak X-omat film (Eastman-Kodak).

Isolation of the cell envelopes and SDS-PAGE of OM proteins. Cell envelopes were isolated (Hirvas *et al.*, 1991b) and their protein pattern was determined by SDS-PAGE (15%, w/v, acrylamide) (Laemmli, 1970). Gels were stained with Coomassie blue.

Computer analyses. Database comparison and manipulation of DNA sequences were performed using the programs contained in the University of Wisconsin Computer Genetics Group Package v. 8 (Madison, WI, USA).

RESULTS

Isolation of supersensitive mutants

E. coli JM105 was mutagenized with diethyl sulfate and cells sensitive to fusidic acid, a hydrophobic antibiotic, were enriched as described in Methods. Survivor cells on LB plates were replica-printed onto fusidic acid plates. Cells not able to grow on replica plates were picked for further studies. Their sensitivity to a set of hydrophobic

Fig. 1. The ratio of LPS to phospholipid biosynthesis at 28 °C, 37 °C and 42 °C for *E. coli* LH530 (○), its parent JM105 (●) and its transductants carrying the plasmids pLH26 (■), pLH29 (▲), pLH30 (△) and pLH31 (□).

and large peptide antibiotics was measured (data not shown). Ability to grow at 42 °C was also tested. About 30 thermosensitive and antibiotic supersusceptible mutants were obtained. Their sensitivity to LPS-specific bacteriophages (C21, Br2, Br60 and Ffm) was similar to that of *E. coli* JM105 indicating no alteration in the LPS core structure. However, the LPS/phospholipid ratio was decreased in most of the mutants (data not shown). One of the mutants, LH530, was chosen for further studies.

Characteristics of *E. coli* LH530

LH530 was sensitive to erythromycin, clindamycin, rifampicin and fusidic acid (Table 1), antibiotics that

Table 1. Susceptibility of LH530 and certain other OM mutants of *E. coli* and their parents to various antibiotics

Antibiotic	MIC (µg ml ⁻¹)						Ratio*		
	JM105	LH530	CDH23-210 (<i>lpxD</i> ⁺)	CDH23-213 (<i>lpxD</i>)	D21	D21f2 (<i>rfa</i>)	JM105/ LH530	CDH23-210/ CDH23-213	D21/ D21f2
Erythromycin	50	1	30	0.50	30	0.75	50	60	40
Clindamycin	30	1	≥ 256	14	≥ 256	3	30	≥ 18	≥ 85
Rifampicin	10	0.125	10	0.016	10	0.25	80	625	40
Fusidic acid	≥ 256	4	≥ 256	16	≥ 256	32	≥ 64	16	8
Vancomycin	≥ 256	24	≥ 256	64	≥ 256	50	≥ 10	4	≥ 5
Gentamicin	1	0.75	1	1	1	0.50	1	1	2
Cefuroxime	2	0.38	3	1	16	6	5	3	3
Ampicillin	1	0.25	3	1	64	12	4	3	5

* Approximate ratio between the MIC for the wild-type parent and that for the corresponding mutant.

Fig. 2. The gene arrangement of the ORF195 chromosomal region and the fragments subcloned into pUC19. Numbers refer to distances in bp. White arrows below the genes indicate the direction of transcription. Black arrows show orientation of the LacZ promoter of the pUC plasmid. Restriction sites: S, *Sau3A*; G, *BglI*; V, *EcoRV*; P, *PstI*; A, *AccI*; H, *SphI*; D, *HindIII*; E, *EcoRI*; and K, *KpnI*. Sites marked with asterisks originate from the vector. Horizontal black bars are used to show entire genes or ORFs present in the plasmid constructions. The insert in pLH32 originates from LH530 whereas the inserts in all the other constructs originate from JM105.

traverse the OM via the hydrophobic pathway of diffusion (Nikaido & Vaara, 1985, 1987). The MICs of these drugs were 30- to 80-fold lower for LH530 than for the parent strain, JM105, and were comparable with the MICs for the heptose-deficient D21f2 mutant (MICs 8- to 85-fold lower) and the *lpxD* mutant CDH23-213 (MICs 16- to 600-fold lower). LH530 was also susceptible to the hydrophilic antibiotics vancomycin (Table 1) and bacitracin (data not shown), which are too large to penetrate the OM through porin channels. No sensitivity or only slight sensitivity was observed to gentamicin and cefuroxime; these antibiotics penetrate the OM through porin channels.

As seen by light microscopy, the cell morphology of LH530 differed from that of the wild-type, and some

cells grew as long filaments at 28 °C and 37 °C. Exponentially growing cells had normal OM protein composition at 28 °C as demonstrated by SDS-PAGE (data not shown).

The ratio of LPS to phospholipid biosynthesis in LH530 and its parent

Bacteria were grown at 28 °C, 37 °C and 42 °C in the presence of radiolabelled acetate, which is incorporated into the fatty acids of phospholipids and LPS. The radioactivities associated with LPS and phospholipids were measured separately, and the ratio of LPS to phospholipid biosynthesis calculated as described in Methods. In the parent strain, JM105, the LPS/phospholipid ratio remained practically the same at all

Table 2. Transcomplementation of *E. coli* LH530, SM101 (*lpxA*), and CDH23-213 (*lpxD*) with various plasmids

Strain	Plasmid	Brief description of insert (complete chromosomal genes present)	MIC ($\mu\text{g ml}^{-1}$)*				Ability to grow at 42 °C
			Fusidic acid	Rifampicin	Erythromycin	Vancomycin	
LH530	pLH29	ORF376, ORF195	250	3	64	> 250	+
LH530	pLH31	ORF195	> 250	8	64	> 250	+
LH530	pLH30	ORF376	4	0.064	1	16	—
LH530	pLH32	ORF195 from LH530	250	4	32	> 250	+
LH530	pGAH317	<i>hlpA</i> , <i>lpxD</i>	8	0.25	2	ND	ND
LH530	pRV40†	<i>lpxA</i>	4	0.25	2	ND	ND
LH530	pLH36‡	ORF376, ORF195 from LH530	128	1.0	64	256	—
LH530	pLH37‡	ORF376, ORF195	128	1.0	24	96	—
CDH23-213	pGAH317	<i>hlpA</i> , <i>lpxD</i>	> 256	6	48	ND	ND
CDH23-213	pLH31	ORF195	8	0.016	0.75	ND	ND
SM101	pRV40†	<i>lpxA</i>	> 250	1.0	8	ND	ND
SM101	pLH31	ORF195	6	0.094	0.50	ND	ND

ND, Not determined.

* MIC determinations were performed at 28 °C (SM101 and CDH23-213) or at 37 °C (LH530) on LB plates containing ampicillin (100 $\mu\text{g ml}^{-1}$).

† Carries *lpxA* in an approximate 6 kb insert from *S. typhimurium* SH5014 (Vuorio *et al.*, 1994).

‡ The inserts were cloned into the low-copy vector pACYC184.

temperatures, but in the mutant the ratio was 75 % at 28 °C, 35 % at 37 °C, and 40 % at 42 °C (nonpermissive temperature) of that in JM105 (Fig. 1).

Qualitative analysis of lipid A of LH530

The lipid A 4'-monophosphate patterns of LH530 grown at 28 °C, 37 °C or 42 °C were identical with that of the parent strain and no lipid A precursors were found in chloroform/methanol extracts of the cells (data not shown).

Complementation of the antibiotic-supersensitive phenotype of the mutant

To identify the gene defective in LH530, we tested the ability of the *E. coli* JM105 chromosomal gene bank to complement the defect. Three plasmids were obtained able to complement the antibiotic susceptibility pattern and the thermosensitive phenotype. Two of the plasmids gave identical restriction patterns. Plasmids with different patterns were chosen for further analysis, and named pLH26 and pLH27 (Fig. 2). Partial restriction mapping showed that they carried inserts approximately 5.5 kb and 5.2 kb in size, respectively. Plasmids shared an overlapping segment of approximately 3.0 kb (Fig. 2). This segment was expected to include the complementing sequence.

An *EcoRI*-*KpnI* fragment, containing the overlapping segment of pLH26 and pLH27, was isolated from pLH27, subcloned into pUC19 and named pLH28 (Fig. 2). Part of the nucleotide sequence of the termini of the

fragment was determined with pUC universal primers. The determined sequence was compared to the sequences in the GenBank database (Burks *et al.*, 1992), by using the FASTA algorithm (Pearson & Lipman, 1988). Sequences derived with forward and reverse primers were 100 % identical with ORF376 and *nikA*, respectively (Sofia *et al.*, 1994). ORF376 and the *nikA* gene are situated close to each other in the 76.0–81.5 min region of the *E. coli* chromosome. This region has been sequenced completely by the *E. coli* Genome Project (Sofia *et al.*, 1994). The only entire ORFs present both in pLH26 and pLH27 are ORF376 and ORF195 (Fig. 2). To see which of them was responsible for complementation, we constructed plasmids pLH29, pLH30 and pLH31. pLH29 carried both ORFs, whereas pLH30 contained only ORF376 and pLH31 only ORF195 (Fig. 2). Plasmids pLH29 and pLH31, but not pLH30, completely reverted the antibiotic-supersensitive phenotype and the thermosensitivity of LH530 (Table 2). The lipid A/phospholipid ratio was similarly restored by pLH26, pLH29 and pLH31, but not by pLH30 (Fig. 1). These results indicate that ORF195 complemented the defect in LH530. Unrelated supersensitive *E. coli* mutants, SM101 (*lpxA*) or CDH23-213 (*lpxD*) were not reverted by pLH31 (Table 2).

ORF195 isolated from JM105 or LH530 in a multi-copy plasmid reverts the mutant phenotype but low-copy expression is insufficient

As shown above, pLH31, carrying the wild-type ORF195 of *E. coli* JM105 in a 1.7 kb *PstI*-*PstI* fragment, was effective in the complementation of the phenotype of

Fig. 3. Northern analysis of ORF195 expression. Total RNA was isolated from exponentially growing cells at 37 °C. Lanes 1–4: 2.5, 5, 10 and 15 µg RNA, respectively, from JM105. Lanes 5–8: 2.5, 5, 10 and 15 µg RNA, respectively, from LH530. The blot was probed with a 300 bp *EcoRV*–*HpaI* ORF195-specific probe, and imaged in a Bio-imagers analyser.

LH530. However, the multi-copy plasmid pLH32 that carried ORF195 from the supersensitive mutant strain LH530 was also effective (Table 2). To determine the complementation ability of ORF195 in a low-copy plasmid, we cloned the 2.4 kb fragment containing ORF376 and ORF195 both from the wild-type and from the mutant strain into a low-copy vector pACYC184. Fragments made blunt-ended were inserted into the *EcoRV* site of the vector. The resulting plasmids were named pLH36 and pLH37. They were introduced into LH530 and sensitivity to antibiotics was tested. With both plasmids, complementation was good at 28 °C (data not shown) and partial at 37 °C (Table 2). Neither could restore the ability of LH530 to grow at 42 °C.

Nucleotide sequence of the wild-type and mutant ORF195

We sequenced the complete 2.0 kb *SphI*–*BglI* insert of pLH31 in both directions, using universal primers and oligonucleotides. This insert contains most of ORF376 and the entire ORF195 of the wild-type strain JM105. The sequence was 100% identical to the published sequence (Sofia *et al.*, 1994). The corresponding restriction fragment derived from the mutant, LH530, was also 100% identical.

Transcript analysis

To determine whether the transcription level of ORF195 was affected in LH530, we performed Northern blot analysis with an ORF195-specific probe using 2.5, 5, 10 and 15 µg total RNA isolated from JM105 and LH530 (Fig. 3). The transcription level of ORF195, quantified as described in Methods, was about two times lower in LH530 than in the wild-type. This, however, could not be entirely responsible for the pleiotropic phenotypic properties of LH530.

DISCUSSION

Thermosensitive, antibiotic-supersusceptible mutants are valuable in studying OM structures and functions. We wanted to isolate a new set of such mutants to reveal

missing parts in the biosynthesis of lipid A. Our strategy was to enrich for fusidic-acid-supersensitive mutants in the presence of penicillin after mutagenization of *E. coli* K-12 by diethyl sulfate. In this way we isolated several mutants that displayed a reduced rate of lipid A synthesis.

One of these mutants, LH530, was shown to be extremely sensitive to a set of hydrophobic antibiotics and large hydrophilic antibiotics that are believed to traverse the OM via the hydrophobic pathway of diffusion. This pathway is practically closed in wild-type strains, but open in certain OM mutants, such as the deep-rough heptose-deficient LPS mutants and lipid A biosynthesis mutants of *E. coli* and *S. typhimurium* (Nikaido, 1990; Nikaido & Vaara, 1987; Hirvas *et al.*, 1991a; Vuorio & Vaara, 1992a, b).

LH530 was thermosensitive and synthesized reduced amounts of lipid A at 37 °C and 42 °C. These properties, as well as antibiotic supersensitivity, are also shown by *E. coli* and *S. typhimurium* strains harbouring mutations in the lipid A biosynthesis genes *lpxA* and *lpxD* (Galloway & Raetz, 1990; Hirvas *et al.*, 1991a; Helander *et al.*, 1992, 1993; Vuorio & Vaara, 1992b). However, the antibiotic sensitivity of LH530 was not reverted by *lpxA* and *lpxD* indicating that it did not harbour mutations in these genes.

At 28 °C and 37 °C some cells of LH530 grew as filaments thus resembling the *E. coli* strains *lpxC* (Normark *et al.*, 1969) and *htrB* (Karow & Georgopoulos, 1991, 1992; Karow *et al.*, 1991). Recently the *htrB* strain has been shown to be deficient in one of the late acyltransferases (the lauroyl transferase) of lipid A (Clementz *et al.*, 1995; Lee *et al.*, 1995). The other late acyltransferase, specific for myristic acid, is encoded by the *msbB* gene (Karow & Georgopoulos, 1992; Clementz *et al.*, 1995; Somerville *et al.*, 1996). Mass spectrometric analyses have shown that the lipid A of both *htrB* and *msbB* mutants contains less myristic acid and lauric acid, respectively, than does the wild-type lipid A (Somerville *et al.*, 1996). In gas chromatographic-mass spectrometric studies we could not detect any change in the fatty acid composition of lipid A of LH530 at 28 °C or 37 °C compared with that of the parent strain (data not shown). Neither could we detect any lipid A precursors in the mutant. However, no precursors have been found in the *lpxA* or *lpxD* mutants either (Galloway & Raetz, 1990; Helander *et al.*, 1992, 1993).

The defect of LH530 was reverted by multiple copies of ORF195. According to the analysis of the sequence data, ORF195 encodes a slightly acidic protein (pI 5.6) with a molecular mass of 21.8 kDa (Sofia *et al.*, 1994). The protein has no amino-terminal signal sequence and no long hydrophobic stretches, suggesting that it may be present in the cytoplasm. ORF195 is not preceded by a Shine–Dalgarno sequence. Most probably it is transcribed from the promoter of ORF376, the gene immediately upstream, as no other putative promoter sequences are present. It is possible that the products of

this operon are regulated together and may also function together. Interestingly, in ORF376 there are several imperfect hexapeptide motifs, characteristic of certain acyl- or acetyltransferases (Vaara, 1992; Vuorio *et al.*, 1994). Immediately 111 bp downstream of the stop codon of ORF195 are the genes for nickel transport, *nikA*, *nikB*, *nikC*, *nikD* and *nikE* (Sofia *et al.*, 1994).

When compared with protein sequences in the SWISS-PROT database, the deduced amino acid sequence of ORF195 showed the best match with *Synechocystis* sp. hypothetical protein (ORF246; Kotani *et al.*, 1994); *Haemophilus influenzae* hypothetical protein (ORF235); a protein encoded by the *lpa-14* gene of *Bacillus subtilis* RB14 (Huang *et al.*, 1993), a possible regulator of the biosynthesis of the lipopeptide antibiotics iturin A and surfactin; and with HetI protein, a possible regulator of growth and development of *Anabaena* sp. (Black & Wolk, 1994). These, furthermore, show homology with proteins encoded by the *sfp*, *psf-1*, *orfX* and *entD* genes from *B. subtilis*, *Bacillus pumilus*, *Bacillus brevis* and *E. coli*, respectively. The function of Sfp and PSF-1 has been suggested to be in the regulation of surfactin biosynthesis or its secretion (Morikawa *et al.*, 1992; Nakano *et al.*, 1992), while EntD has been suggested to be involved in the production or secretion of the peptidic siderophore enterobactin (Armstrong *et al.*, 1989; Grossman *et al.*, 1993). The function of the *orfX* product is not known, but it is located in an operon involved in the biosynthesis of the cyclic lipopeptide gramicidin (Krättschmar *et al.*, 1989). Thus, *hetI* affects heterocyst formation, *lpa-14*, *sfp* and *psf-1* the synthesis or secretion of lipopeptide antibiotics and *entD* siderophore secretion. Common to all these functions is that they are induced under nutrient deprivation. However, the homology of the ORF195 product with the *lpa-14* gene product and HetI is relatively low, 37.7% identity in an 106 amino acid overlap and 29.2% identity in an 154 amino acid overlap, respectively.

Interestingly, a perfect match with a six amino acid long consensus sequence, FNI [L]SHS, is present in ORF195 of *E. coli*, SFP and LPA-14 of *B. subtilis*, ORF235 of *H. influenzae*, ORFX of *B. brevis*, HetI of *Anabaena* sp., and PSF-1 of *B. pumilus*. The *entD* gene product of *E. coli* and ORF246 of *Synechocystis* sp. have sequences that partially match the consensus, GSISHCG and FNVAHSG, respectively. Whether this consensus indicates related functions of these proteins remains to be elucidated. The modification of the histidine residue in the consensus region of ORF195 is underway in our laboratory.

According to the sequencing data, ORF195 in LH530 does not harbour any mutations. This is confirmed by the similar complementation ability of ORF195 isolated from either JM105 or LH530. Thus, ORF195 seems to suppress a defect caused by some other gene. The suppression is accessed only if ORF195 is expressed in a multi-copy plasmid. Whether ORF195 is a *msbA*- or *accBC*-like suppressor of the *htrB* mutant (Karow *et al.*,

1992; Karow & Georgopoulos, 1993) could be determined by suitable complementation studies. We are currently searching an *E. coli* gene bank for the gene corresponding to the one mutated in LH530. This gene may also be lethal when expressed from a multi-copy plasmid, and thus the preparation of a low-copy vector gene bank is being considered.

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