The outer membrane of lipid A-deficient *Escherichia coli* mutant LH530 has reduced levels of OmpF and leaks periplasmic enzymes

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We have previously described a new *Escherichia coli* K-12 mutant, LH530, which has a defective outer membrane. LH530 is very sensitive to hydrophobic antibiotics, does not grow at 42 °C and synthesizes reduced amounts of lipid A. Phenotypically LH530 is very similar to the known lipid A biosynthesis mutants of *E. coli* and *Salmonella typhimurium*. Its genetic defect is not known, but the defect is suppressed by multiple copies of ORF195. Here we show that at 37 °C LH530 contains a reduced amount of the OmpF porin and that it leaks periplasmic β -lactamase at 37 °C and 42 °C. We further show that ORF195, when present at low copy number, restores the antibiotic resistance and lipid A biosynthesis of LH530 at 28 °C, but not at higher temperatures. In contrast, OmpF expression is restored at 37 °C.

Keywords: *Escherichia coli*, ORF195, OmpF, periplasmic enzymes, outer membrane permeability

INTRODUCTION

Lipopolysaccharide (LPS) is an essential component of the outer membranes (OMs) of Gram-negative bacteria, and mutations affecting the structure of LPS severely decrease the ability to grow in normal environments (Nikaido & Vaara, 1987). Defects in the LPS inner core region render the OM permeable to hydrophobic antibiotics and sensitize the bacteria to these agents. However, mutations affecting the early phase of lipid A biosynthesis are lethal to the bacteria. Thus, leaky or conditional, e.g. thermosensitive, lipid A mutants are the best choice for studying the lipid A biosynthesis pathway. At permissive temperatures lipid A mutants are even more sensitive to hydrophobic antibiotics than the mutants that are defective in synthesis of the inner core part of the LPS (Vaara, 1993). Moreover, at non-permissive temperatures lipid A deficiency leads to leakage of periplasmic enzymes (Vuorio & Vaara, 1992).

The proper trimerization and export of porin proteins requires a specific LPS structure (Sen & Nikaido, 1991) and deep rough mutants contain reduced amounts of OM proteins (Ames *et al.*, 1974; for review, see Nikaido & Vaara, 1985). The relative abundance of OmpC and OmpF is a function of the temperature and osmolarity

of the growth medium (Pratt *et al.*, 1996). *envZ* and *ompR* control porin gene transcription in response to osmolarity, and the antisense RNA, *micF*, influences *ompF* translation. OmpF deficiency can also result from *dsbA* mutations (Pugsley, 1993) and pleiotropic *tolC* mutations (Misra & Reeves, 1987; Forst & Inouye, 1988). DbsA is a periplasmic disulphide oxidoreductase responsible for the formation of disulphide bonds in periplasmic proteins and in periplasmic domains of inner and outer membrane proteins as well as secreted proteins (Bardwell, 1994). TolC probably affects OmpF levels by increasing *micF* expression.

Recently, we isolated a new *E. coli* K-12 mutant, LH530 (Hirvas *et al.*, 1997), which in many aspects resembles the known lipid A biosynthesis mutants. It is hypersusceptible to hydrophobic antibiotics, grows in a heat-sensitive manner and has a decreased rate of lipid A biosynthesis. The defect is suppressed by multiple, but not by low copy numbers, of ORF195 (Hirvas *et al.*, 1997), an ORF located in the 76 min region of the chromosome (Sofia *et al.*, 1994).

In this work we show that LH530 leaks periplasmic β -lactamase and is deficient in OmpF protein at higher temperatures. Further we compare the effect of low- and high-copy expression of ORF195 in the mutant on the rate of lipid A biosynthesis and on the expression of OmpF.

Abbreviation: OM, outer membrane.

METHODS

Bacterial strains and plasmids and general DNA manipulations. The OM mutant LH530 originates from JM105 (Yanisch-Perron *et al.*, 1985) and has been described previously (Hirvas *et al.*, 1997). DME553 is an *ompF* deletion mutant of *E. coli* K-12 (Misra & Benson, 1988). The pUC19-derived plasmids, pLH29 (containing ORF376 and ORF195) and pLH31 (containing ORF195), as well as the low-copy-number vector pACYC184-derived plasmid pLH37 (containing ORF376 and ORF195) have been described previously (Hirvas *et al.*, 1997). General DNA manipulations were carried out as described by Sambrook *et al.*, (1989).

Measurement of leakage of periplasmic enzymes and lipid A biosynthesis rate. The leakage of β -lactamase and glucose-6-phosphate dehydrogenase from bacteria was measured essentially as described by Vuorio & Vaara (1992) using the methods of O'Callaghan *et al.* (1972) and Langdon (1966), respectively.

The ratio of LPS to phospholipid biosynthesis in LH530 and its derivatives was assayed (Vuorio & Vaara, 1995; Hirvas *et al.*, 1997) after labelling their fatty acids with radiolabelled acetate *in vivo* at 28 °C, 37 °C and 42 °C.

Analysis of major OM proteins. LH530, JM105 and DME 553 (*ompF*⁻) were grown on L-plates at 28 °C and 37 °C. Plasmid-containing derivatives of LH530 were grown on L-plates containing 100 μ g ampicillin ml⁻¹ or 30 μ g chloramphenicol ml⁻¹. Approximately 10¹⁰ bacteria were suspended in 10 ml

0.9% NaCl and collected by centrifugation. Bacterial cell envelopes were isolated (Hirvas *et al.*, 1991a) and their protein profiles analysed in 10% SDS-PAGE (Laemmli, 1970). Gels were stained with Coomassie Blue, scanned by an Agfa Arcus II scanner and edited by Canvas 3.5 using a Macintosh 7500/100 computer.

RESULTS

Release of periplasmic enzymes

We have previously shown that the OM of LH530 is permeable to hydrophobic agents (Hirvas *et al.*, 1997). Here we studied whether its OM leaks periplasmic proteins, as does the OM of certain lipid A mutants. We therefore introduced pUC19 (encoding periplasmic β -lactamase) into LH530. As a control we introduced plasmid pLH31 into LH530. pLH31 is a pUC19 derivative and contains wild-type ORF195. A temperature shift from 28 °C to 37 °C or 42 °C induced approximately 39% and 45% leakage of the periplasmic β -lactamase from LH530(pUC19), respectively. With pLH31 the leakage was only 3.5% and 1.0%, respectively (Fig. 1a, b). At 28 °C the leakage was less than 12% (data not shown). The cytoplasmic marker enzyme, glucose-6-phosphate dehydrogenase was not released (Fig. 1c, d).

Fig. 1. Release of periplasmic β -lactamase and cytoplasmic glucose-6-phosphate dehydrogenase from derivatives of LH530. LH530 carrying either pUC19 (□, ■) or ORF195 (○, ●) was shifted from 28 °C to 37 °C (a, c) or to 42 °C (b, d) at time zero. β -Lactamase (a, b) and glucose-6-phosphate dehydrogenase (c, d) activities in the growth medium (●, ■) and the corresponding cellular activities (○, □) are shown.

Fig. 2. The ratio of LPS to phospholipid biosynthesis (PL) at 28 °C, 37 °C and 42 °C in LH530 (○), its parent JM105 (●), and its transformants carrying the multi-copy-number plasmid pLH29 (■) or the low-copy-number plasmid pLH37 (□).

Fig. 3. SDS-PAGE of cell envelopes of JM105 and LH530 and its derivatives. Lanes: 1, 2, JM105; 3, 4, LH530; 5, 6, LH530 with multi-copy-number plasmid pLH29; 7, 8, LH530 with low-copy-number plasmid pLH37, each grown at 28 °C and 37 °C, respectively; 9, DME553.

The ratio of lipid A to phospholipid biosynthesis of LH530 harbouring ORF195 in low- or multi-copy-number plasmids

The low-copy-number plasmid, pLH37, was able to restore the ratio of lipid A to phospholipid biosynthesis in LH530 (Fig. 2) at 28 °C, but not at 37 °C and 42 °C, where the ratio was about 50% and 42% of that of JM105, respectively. In our previous publication we showed that pLH37 also restores the antibiotic resistance of LH530 at 28 °C, but not at 37 °C and 42 °C. In contrast the multi-copy-number plasmid, pLH29, restores the ratio at all temperatures (Hirvas *et al.*, 1997). Thus LPS levels correlate with the antibiotic susceptibility of LH530 and its derivatives.

The protein composition of LH530

Fig. 3 shows the OM protein profile of LH530 and its derivatives. At 37 °C the profile of LH530 (lane 4) differed markedly from that of JM105 (lane 2) and resembled the profile of the OmpF-deficient strain DME553 (lane 9). The OmpC content was compensatorily increased. Both the multi-copy-number

plasmid (pLH29) and low-copy-number plasmid (pLH37) restored the protein profile to one similar to JM105. At 28 °C the OM protein profiles of JM105 (lane 1), LH530 (lane 3), and pLH29- (lane 5) and pLH37- (lane 7) containing derivatives of LH530, were identical.

We have previously shown that high-copy expression of ORF195 (i.e. as in pLH29) is needed to suppress the defect of LH530 at all temperatures. Low-copy expression (pLH37) suppresses the defect totally at 28 °C, and almost totally at 37 °C, but does not restore the ability of LH530 to grow at 42 °C. Fig. 3 shows that both pLH29 and pLH37 were able to restore the OmpF content of LH530 at 37 °C (lanes 6 and 8, respectively).

DISCUSSION

In this work we have further characterized the thermo-sensitive and lipid A-deficient mutant, LH530, and showed that at 37 °C its OM is deficient in OmpF porin and leaks periplasmic β -lactamase. We also demonstrated that at 37 °C the low-copy expression of ORF195 (the high-copy suppressor gene of LH530) only partially restores the rate of lipid A biosynthesis of LH530 to normal levels. However, it does restore its OmpF level.

In many respects LH530 resembles the well-characterized lipid A mutants *lpxA* and *lpxD* (Galloway & Raetz, 1990; Hirvas *et al.*, 1991b; Vaara, 1993; Helander *et al.*, 1992, 1993; Vuorio & Vaara, 1992) in being sensitive to hydrophobic antibiotics, in producing decreased amounts of LPS (75% at 28 °C, 35–40% at 37 °C and 42 °C; Hirvas *et al.*, 1997) and in leaking periplasmic proteins. In addition we have demonstrated that the *lpxD* mutant is OmpF deficient at 37 °C (unpublished observation) similar to LH530. However, LH530 does not harbour any mutation in *lpxA* or *lpxD* (Hirvas *et al.*, 1997).

Our results indicate that the synthesis, assembly, or translocation of OmpF is dependent on certain level of LPS synthesis. Although some studies show that OmpA assembly differs from that of OmpF and OmpC (Ried *et al.*, 1990; Bocquet-Pagès *et al.*, 1981), it is difficult to explain why only OmpF is affected in LH530. A similar situation exists in certain deep rough mutants (for review, see Nikaido & Vaara, 1985). However, another explanation for the OmpF deficiency could be that, among other periplasmic components, LH530 also leaks membrane-derived oligosaccharides as well as disulphide oxidoreductase encoded by *dsbA*. Mutations in *dsbA* have been shown to decrease OmpF production in *E. coli* (Pugsley, 1993), and membrane-derived oligosaccharides have been proposed to have a role in recognition of the growth medium osmolarity (Pratt *et al.*, 1996).

The OmpF deficiency is unlikely to be responsible for the antibiotic hypersensitivity of LH530, as it is sensitive even at 28 °C, where its OmpF level is normal. Furthermore, *E. coli* and *S. typhimurium* mutants lacking one or more of the porins or the OmpA protein, have an

almost unaltered sensitivity to hydrophobic antibiotics (Chai & Foulds, 1977; Lounatmaa & Nurminen, 1977; van Alphen *et al.*, 1977). The sensitivity of LH530 to hydrophobic antibiotics could be similar to that of deep rough LPS mutants, in which the relative lack of LPS in OM results in the compensatory appearance of glycerophospholipids in the outer leaflet of OM (for review, see Nikaido & Vaara, 1985). As LH530 is also sensitive to large hydrophilic antibiotics, transient rupturing (and resealing) of the OM may be involved, as is also suggested for some lipid A biosynthesis mutants (Vaara, 1993).

Obviously, LH530 has a mutation in a gene essential in OM biosynthesis. Phenotypically the mutant resembles the known lipid A biosynthesis mutants of *E. coli* and *S. typhimurium* (Galloway & Raetz, 1990; Hirvas *et al.*, 1991b; Helander *et al.*, 1992, 1993; Vuorio & Vaara, 1992; Normark *et al.*, 1969; Karow & Georgopoulos, 1991, 1992; Karow *et al.*, 1991). In contrast to mutants deficient in late acyl transferases (Lee *et al.*, 1995; Clementz *et al.*, 1995), there is no detectable change in the fatty acid composition of lipid A of mutant LH530 (Hirvas *et al.*, 1997).

The mutation in LH530 does not necessarily involve any of the so far uncharacterized structural genes of lipid A biosynthesis. Thus, the characterization of mutant LH530 and the role of its suppressor gene, ORF195, in membrane assembly might give new insights into the regulation of the biosynthesis pathway for lipid A or into the translocation and biogenesis of OM components.

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