Inhibition of lipid biosynthesis induces the expression of the *pspA* gene

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Treatment of *Escherichia coli* with diazaborine strongly induces the synthesis of a 28 kDa protein which is associated with the cytoplasmic membrane. The partial amino acid sequence proved that this protein is identical to the phage shock protein PspA. The kinetics of the expression of the *pspA* gene were determined in an *E. coli* strain which carried a *pspA-lacZ* fusion in the chromosome. PspA synthesis is independent of the growth phase. It is, however, strongly induced when fatty acid biosynthesis is inhibited by diazaborine or cerulenin. Treatment with either compound also causes dose-dependent inhibition of phospholipid biosynthesis whose degree correlates with the induction of PspA. Another cause of induction of PspA synthesis is treatment of *E. coli* with globomycin, which is an inhibitor of the processing of lipoproteins.

Keywords: *Escherichia coli*, *pspA* gene, lipid synthesis, diazaborine, globomycin

INTRODUCTION

Incubation of *Escherichia coli* cells with diazaborines, a group of boron-containing antibacterial compounds (Grassberger *et al.*, 1984), leads to pleiotropic effects in the biosynthesis and composition of membrane constituents, i.e. inhibition of fatty acid-, phospholipid- and lipopolysaccharide biosynthesis (Turnowsky *et al.*, 1989; Högenauer & Woisetschlager, 1981). A specific effect on cell morphology that results in a retraction of the cytoplasmic membrane, predominantly at the poles of the treated cells, was also observed. The target of diazaborines is the *envM* gene product (Turnowsky *et al.*, 1989), which we have recently shown to be the NADH-dependent enoyl-ACP reductase of *E. coli* (Bergler *et al.*, 1994). Since the enzymic activity of the EnvM protein has been identified the corresponding gene has been renamed *fabI* (Bergler *et al.*, 1994). When the *E. coli* mutant JP1111, which carries a temperature-sensitive *fabI* allele (Egan & Russell, 1973), was incubated at the non-permissive temperature, similar effects on lipid biosynthesis and membrane morphology were observed. In addition, treatment of wild-type *E. coli* cells with the diazaborine derivative 2b18 (Grassberger *et al.*, 1984) at 30 °C or incubation of the *fabI* mutant at the non-permissive temperature strongly induced the accumulation of a protein with an apparent molecular mass of 28 kDa that is associated with the cytoplasmic membrane (Turnowsky *et al.*, 1989).

In order to study the relationship of FabI to the 28 kDa protein, we purified this protein and determined its partial amino acid sequence. We now show that this protein is identical to the recently described PspA protein (Brisette *et al.*, 1991). PspA is induced in *E. coli* cells under extreme stress conditions, such as treatment with heat or ethanol, or by infection with filamentous bacteriophages (Brisette *et al.*, 1990). Recently it was shown that PspA stimulates efficient protein export in *E. coli* (Kleerebezem & Tommassen, 1993) and that expression of *pspA* is also induced under conditions that are known to interfere with protein translocation (Kleerebezem & Tommassen, 1993; Carlson & Silhavy, 1993). It is, however, a non-essential protein whose function in the cell under uninduced conditions is not fully understood. Inhibition of the *fabI* gene product by diazaborine is a novel cause for the induction of PspA. The expression of the protein correlates with inhibition of lipid biosynthesis.

METHODS

Bacterial strains, plasmids, and phages. Strains, plasmids, and phages are listed in Table 1. *E. coli* was grown in either 2 × TY medium (Sambrook *et al.*, 1989), in 2 × TY medium with a low salt concentration (0.05 % w/v), or in Minimal Medium A (Miller, 1972). Antibiotics were added when needed at the following concentrations: epicillin, 150 µg ml⁻¹; kanamycin, 40 µg ml⁻¹. For cloning procedures pUC118 (Vieira & Messing, 1987) was used. Other plasmids are listed in Table 1.

Chemicals and enzymes. Diazaborine, compound 2b18, was

Table 1. Bacterial strains, plasmids and phages

Strain	Genotype, description or characteristics	Source or reference
<i>Escherichia coli</i>		
MC4100	<i>F</i> ⁻ <i>araD139</i> Δ(<i>argF-lac</i>) <i>U169 rpsL150 relA1 ffbB5301 deoC1 ptsF25 rbsR</i>	T. Silhavy, Princeton, NJ, USA
XL1	<i>endA1 usdR17(bsdR⁻, bsdM⁺) supE44 thi-1 recA1 gyrA96 relA1 Δ(lac)</i> [F' <i>proAB lacI^qZΔ</i> M15, Tn10 (Tet ^R)]	Stratagene
JP1111	<i>Hfr galE45 λ⁻ envM329(ts) relA1 spoT1</i>	B. Bachmann, <i>E. coli</i> Genetic Stock Center, Yale University
K12 5K	<i>F</i> ⁻ <i>tre thi rpsL⁺ bsdR⁻ bsdM⁺ lac thr λ⁻</i>	W. Goebel, Universität Würzburg, FRG
MC1061	<i>bsdR2 bsdM⁺ bsdS⁺ araD139 Δ(ara-leu)₇₆₉₇ Δ(lac)_{x74} galE15 galK16 rpsL(Str^R) mcrA mcrB1</i>	Casadaban & Cohen (1980)
MC3	MC4100 with integrated λφAD3, <i>lac</i> ⁺	This work
Plasmid		
pRS591	Ap ^R , <i>lacZ'</i> Y A <i>ori</i>	Simons <i>et al.</i> (1987)
R1-16	Km ^R	Sandoz-Forschungsinstitut, Vienna
pAD1	Ap ^R , 4.5 kb <i>EcoRI</i> fragment from λ 1C2, carrying the <i>psp</i> operon, in pUC118	This work
pAD2	Ap ^R , 529 bp <i>EcoRI</i> fragment (PCR amplified from pAD1) in pUC118	This work
pAD3	Ap ^R , 529 bp <i>EcoRI</i> fragment (PCR amplified from pAD1) in pRS591	This work
Phages		
λ RS45	Phage vector for the construction of single copy <i>lacZ</i> -fusion prophages, <i>lac</i> ⁻	Simons <i>et al.</i> (1987)
λ 1C2	Recombinant λ phage carrying the <i>psp</i> operon	Kohara <i>et al.</i> (1987)
M13	Filamentous ssDNA phage	
M13KO7	Km ^R , p15A <i>ori</i>	Vieira & Messing (1987)
λφAD3	Ap ^R , <i>lac</i> ⁺ ; recombinant of λ RS45 and pAD3 carrying the <i>lacZ</i> -fusion of pAD3	This work

described by Grassberger *et al.* (1984). Epicillin was from Biochemie. Kanamycin and cerulenin were from Sigma. Nalidixic acid was purchased from Serva. Globomycin was a gift of Sankyo. Radiochemicals were obtained from Dupont-NEN. Enzymes for DNA work were from Boehringer GmbH and New England Biolabs Inc. For oligonucleotide synthesis the reagents were purchased from Applied Biosystems.

DNA work. Recombinant techniques were performed using the methods of Ausubel *et al.* (1987), Sambrook *et al.* (1989), or the protocols of the manufacturers. Transformations were done with the standard CaCl₂ protocol. DNA from the recombinant phage λ1C2 (Kohara *et al.*, 1987) was isolated according to Silhavy *et al.* (1984) with *E. coli* K12 5K as the host. Single-stranded DNA was prepared from recombinant plasmids derived from pUC118 with the helper phage M13KO7 (Vieira & Messing, 1987). The sequence of the PCR-amplified DNA fragment was determined in the single-stranded form by the chain termination method of Sanger *et al.* (1977) using a Sequenase Kit (USB).

Cloning procedures. The *pspA* operon on a 4.5 kb *EcoRI* fragment from the recombinant phage λ1C2 (Kohara *et al.*, 1987) was subcloned into appropriately cleaved pUC118. The recombinant plasmid was designated pAD1. The identity of *pspA* was confirmed by Southern hybridization of restriction enzyme digests of λ1C2 DNA with the 5'-endlabelled synthetic oligonucleotide PSP A2 as a probe according to standard procedures (Ausubel *et al.*, 1987). The regulatory region of the *psp* operon was amplified from pAD1 as a 539 bp DNA fragment using PCR. Two specific oligonucleotides were synthesized

according to the sequence published by Brissette *et al.* (1991). PSP A1 contains nucleotides 1 to 29' of the *psp* operon (5' TAGCGAATTTCATCAAGAAATAGCGTACCG 3'). PSP A2 is complementary to nucleotides 517 to 539 which are located within the coding region of *pspA* (5' GTTGGGAATTCACGA-TGTCGGCAA 3'). Both oligonucleotides contained a single base exchange in order to create an *EcoRI* restriction site to facilitate subsequent cloning; the exchanged bases are underlined. The PCR reaction was done with *Pfu* DNA polymerase (Stratagene) in a BIO-MED thermal cycler. The PCR product was purified from agarose gels with the US Bioclean MP Kit, cleaved with *EcoRI* and ligated into the appropriately cut pUC118 as well as the *lacZ* fusion vector pRS591 (Simons *et al.*, 1987). The resulting recombinant plasmids were designated pAD2 and pAD3, respectively.

Construction of a single copy *lacZ* fusion. The plasmid-borne *lacZ* fusion pAD3 was transferred to the λ phage vector λRS45 (Simons *et al.*, 1987) by homologous recombination. *E. coli* MC4100, carrying the multi-copy plasmid pAD3, was used as a host for the growth of λRS45. Recombinant phage were isolated from individual plaques on the basis of a Lac⁺ phenotype on X-Gal plates. The lysate was used to infect *E. coli* MC4100 (Silhavy *et al.*, 1984). Lysogens with the *pspA-lacZ* fusion in the chromosome were selected as Lac⁺ colonies on X-Gal plates and were designated *E. coli* MC3.

Phospholipid biosynthesis. *E. coli* K12 5K was grown in phosphate-free medium (Garen & Levinthal, 1960) to early exponential phase (OD₆₀₀ 0.3). After addition of 10 μCi of [³²P]orthophosphate the cultures were treated with cerulenin

(50 or 100 $\mu\text{g ml}^{-1}$) at 30 °C. At various time points the cells were harvested and extracted as described by Turnowsky *et al.* (1989). The radioactivity contained in aliquots of the chloroform phases was determined using scintillation spectroscopy.

Conjugation. The donor strain *E. coli* MC1061 carrying the plasmid R1-16 was mated for 45 min at 37 °C with the recipient strain *E. coli* MC3. The ratio of donor to recipient was 1:10. After vigorous shaking, aliquots of the mating mixture were plated on X-Gal plates containing 40 $\mu\text{g kanamycin ml}^{-1}$. The transconjugants were selected for kanamycin resistance and Lac⁺ phenotype and designated *E. coli* MC3 R1-16.

β -Galactosidase activity. *E. coli* MC3 was grown in Minimal Medium A (Miller, 1972) at 30 °C to early exponential phase (OD_{600} 0.2–0.3). The cultures were then treated with either diazaborine at 4 and 20 $\mu\text{g ml}^{-1}$, cerulenin at 50 and 100 $\mu\text{g ml}^{-1}$, globomycin at 10 and 50 $\mu\text{g ml}^{-1}$, nalidixic acid at 40 $\mu\text{g ml}^{-1}$, hydrogen peroxide at 70 $\mu\text{g ml}^{-1}$, or naphthochinon at 100 $\mu\text{g ml}^{-1}$. At various times the cultures were sampled and the β -galactosidase activity in the aliquots was determined according to Miller (1972). The activity was determined in duplicates and each experiment was performed at least two times. Results of representative experiments are shown. In the case of the infection with the filamentous phage M13, *E. coli* MC3 R1-16 was infected in early exponential phase with an M13 lysate at a multiplicity of 10 and the β -galactosidase activity determined at different times after infection as above.

Purification of the 28 kDa protein. The conditional lethal *fabI* mutant *E. coli* JP1111 was grown in low salt 2 × TY medium to early exponential phase (OD_{600} 0.3). Subsequently the temperature was shifted to 42 °C for 5 h. The cells were harvested by centrifugation and 4.5 g (wet weight) of cells were broken by sonication. The cell envelope fraction was collected by centrifugation for 60 min at 48000 *g* and extracted with 50 mM Tris/HCl, pH 7.6, containing 2.5% (w/v) Triton X-100 for 30 min at room temperature. Insoluble material was removed and the supernatant fraction diluted with 50 mM Tris/HCl, pH 7.6, to give a final Triton X-100 concentration of 0.5% (w/v). All buffers for the subsequent purification steps contained 0.5% (w/v) Triton X-100. Purification of the 28 kDa protein was performed by fast protein liquid chromatography (FPLC, Pharmacia) using the following procedure. Aliquots of the supernatant, containing approximately 1 mg total protein, were applied to a Mono Q column which had been equilibrated with 50 mM Tris/HCl buffer, pH 7.6, and the proteins were eluted with a linear salt gradient of 0–1 M LiCl in 50 mM Tris/HCl, pH 7.6. Individual fractions were analysed on a 11% polyacrylamide–SDS gel. The 28 kDa protein eluted between 350 and 375 mM LiCl. Fractions containing the protein were pooled and dialysed against 50 mM sodium acetate buffer, pH 5.1. Under these conditions the protein precipitated. The precipitate was collected by centrifugation and washed twice with 50 mM sodium acetate buffer, pH 5.1. The pellet was finally dissolved in 50 mM Tris/HCl, pH 7.6, 0.5% Triton X-100. After incubation for 60 min at 37 °C the purified protein was unstable and cleaved into four distinct polypeptides. One of the cleavage products was subjected to amino acid sequence analysis.

Protein analysis. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as a standard. Cytoplasmic membrane proteins were prepared as described earlier (Turnowsky *et al.*, 1989), except that the growth medium was 2 × TY with low salt content. Proteins were separated on 11% polyacrylamide–SDS gels and stained with Coomassie Brilliant Blue.

RESULTS

Induction, purification, and identification of the 28 kDa protein

Incubation of the temperature-sensitive *fabI* mutant *E. coli* JP1111 at the non-permissive temperature or with diazaborine at 30 °C leads to the inactivation of the FabI protein and the cessation of cell growth (Turnowsky *et al.*, 1989). This effect is accompanied by the induction of a protein with an apparent molecular mass of 28 kDa which is associated with the cytoplasmic membrane (Turnowsky *et al.*, 1989; and Fig. 1, lanes 2 and 3). The induction is progressive and most prominent after 4 to 6 h incubation. Nalidixic acid induces the synthesis of conventional heat-shock proteins. However, it did not induce the 28 kDa protein, nor did it inhibit the induction by diazaborine (data not shown). The specific correlation between inhibition of FabI and induction of the 28 kDa protein prompted us to purify this protein. Cell envelope fractions of *E. coli* JP1111, grown at the non-permissive temperature for 5 h, were treated with Triton X-100 to solubilize cytoplasmic membrane proteins. Aliquots were applied to a Mono Q column and the proteins eluted with

Fig. 1. Induction and purification of the PspA protein. Cytoplasmic membrane proteins isolated from the *fabI* ts mutant *E. coli* JP1111 which was grown at 30 °C (lane 1), at 30 °C in the presence of 20 $\mu\text{g diazaborine ml}^{-1}$ for 5 h (lane 2), and at 42 °C for 5 h (lane 3) were separated on an 11% polyacrylamide–SDS gel and stained with Coomassie Brilliant Blue. Lanes 4 and 5 show the enrichment of the 28 kDa protein after FPLC-chromatography on a Mono Q column (lane 4) and after precipitation at pH 5.1 (lane 5). The positions of the molecular mass standards and the 28 kDa PspA protein are indicated. Total protein loaded on the gel was: 35 μg on lanes 1 to 3, 20 μg on lane 4, and 15 μg on lane 5.

a linear gradient of lithium chloride as described in Methods. Individual fractions were analysed by SDS-PAGE and those containing the 28 kDa protein were pooled (Fig. 1, lane 4) and dialysed against sodium acetate buffer. At the acid pH of the buffer the 28 kDa protein precipitated preferentially and, therefore, was enriched manyfold upon resuspension (Fig. 1, lane 5). The purified protein was unstable during incubation at 37 °C, probably due to a contaminating protease, and could be resolved into four distinct polypeptides by SDS-PAGE (data not shown). The N-terminal amino acid sequence of one of the cleavage products was determined by Edman degradation and was found to be identical to amino acids 60–79 of the sequence of the PspA protein of *E. coli*, which was published by Brissette *et al.* (1991). Although the derived molecular mass of PspA is 25·562 kDa (Brissette *et al.*, 1991) the protein showed an apparent molecular mass of approximately 28 kDa in our SDS gel system.

Cloning of the *E. coli* *psp* operon and construction of the *pspA*–*lacZ* fusion

The *psp* operon has been mapped by P1 transduction and found to be closely linked to *pyrF* and *rnb* between min 28·3 and 29 of the *E. coli* chromosome (Brissette *et al.*, 1991). Based on these data the λ phage 1C2 from the collection of Kohara *et al.* (1987) was expected to contain the *psp* operon. Phage DNA was prepared by standard procedures and cleaved with various restriction enzymes. The DNA fragments were separated on agarose gels and analysed by Southern hybridization. As a probe the specific oligonucleotide PSP A2 was used, which had been synthesized according to the published sequence of the *psp* operon. The 4·5 kb *EcoRI* fragment of the DNA from the recombinant λ phage 1C2 hybridized with the oligonucleotide and thus carried the *psp* operon. The exact location of the operon could therefore be determined and has been assigned to coordinate 1384 of the Kohara *et al.* (1987) *E. coli* chromosomal map. The 4·5 kb *EcoRI* fragment, illustrated in Fig. 2, was cloned into the appropriately cleaved vector pUC118 and the recombinant plasmid designated pAD1. *E. coli* JP1111 cells that carried this multi-copy plasmid expressed the PspA protein to a high level when treated with diazaborine. The electrophoretic mobility was identical to that of the protein which was induced by incubation of plasmid-free *E. coli* cells with diazaborine (Fig. 3, lanes 1 and 2). Under non-inducing conditions PspA was only moderately expressed from pAD1 (Fig. 3, lane 3), probably because of the negative autoregulatory activity of PspA (Weiner *et al.*, 1991).

The putative promoter region upstream of *pspA* was amplified by PCR with the use of the two specific primers PSP A1 and PSP A2 (Fig. 2). Single base exchanges in the sequences of both primers created *EcoRI* restriction sites which were used for subsequent cloning steps. The 539 bp PCR product was cleaved with *EcoRI* and ligated into the *lacZ* fusion vector pRS491 (Simons *et al.*, 1987). The recombinant plasmid with the insert in the correct orientation was designated pAD3. This construction

Fig. 2. Section of the physical map from 1340 to 1390 kb of the *E. coli* chromosome as described by Kohara *et al.* (1987). The locations of the *fabI* gene and the *psp* operon are indicated by arrows. 1C2 designates the recombinant λ phage from the Kohara *et al.* (1987) collection that carries the *psp* operon. The 4·5 kb *EcoRI* fragment that hybridized to the synthetic oligonucleotide PSP A2 is shown in grey. The construction of pAD1 which comprised the 4·5 kb *EcoRI* fragment with the *psp* operon in pUC118 is illustrated below the restriction map. The insert DNA is shown as a grey bar. Below, the organization of the *psp* operon as published by Brissette *et al.* (1991) is shown. The putative promoter is marked with P and the orientation of transcription with an open arrowhead at the bottom of the figure. PSP A1 and PSP A2 are the specific oligonucleotides used for the amplification of the 539 bp fragment containing the *pspA* promoter region.

contains the region spanning nucleotide 6 to nucleotide 530 of the DNA sequence of the *psp* operon including the promoter region of the *pspA* gene as published by Brissette *et al.* (1991). The nucleotide sequence of the PCR product was determined and was found to be identical to the published sequence except for the two base pair exchanges which had been introduced to create the *EcoRI* restriction sites for cloning purposes. *E. coli* cells containing the *pspA*–*lacZ* fusion on the multi-copy plasmid pAD3 expressed extremely high β -galactosidase activity in the range of 8000 units, according to the method of Miller (1972). In order to assess the *pspA* promoter activity independently of the high copy number of pAD3 the *pspA*–*lacZ* fusion was introduced into the *E. coli* chromosome as a single copy by infection with the recombinant phage $\lambda\phi$ AD3 (Simons *et al.*, 1987). The lysogen that carried the *pspA*–*lacZ* fusion at the λ attachment site *attP* in the chromosome was designated *E. coli* MC3. Chromosomal DNA of the lysogen MC3 was analysed by Southern hybridization with both a *lacZ*-specific and a *pspA*-promoter specific probe. The result

Fig. 3. Expression of the PspA protein from the recombinant plasmid pAD1. Cytoplasmic membrane proteins isolated from *E. coli* JP1111 treated with 20 μg diazaborine ml^{-1} for 5 h at 30 $^{\circ}\text{C}$ (lane 1), from *E. coli* JP1111 pAD1 grown in the presence of 20 μg diazaborine ml^{-1} (lane 2), and *E. coli* JP1111 pAD1 grown at 30 $^{\circ}\text{C}$ under non-inducing conditions (lane 3) were separated by SDS-PAGE on an 11% gel and stained with Coomassie Brilliant Blue. In lane 4 the extracts from lanes 1 and 2 were mixed to prove the identity of the proteins encoded by the chromosomal gene and the recombinant plasmid, respectively. The section of the stained polyacrylamide gel containing PspA is shown. The positions of two molecular mass markers are indicated. The position of PspA is marked with an arrow.

indicated that the *pspA-lacZ* fusion was integrated in the chromosome of *E. coli* MC3 as a single copy.

Induction of PspA by diazaborine treatment

The β -galactosidase activity of the *pspA-lacZ* fusion strain *E. coli* MC3 was around 100 units according to Miller (1972) and was independent of the growth phase (Fig. 4). When identical cultures of *E. coli* MC3 were incubated to early exponential phase at 30 $^{\circ}\text{C}$, treated with 4 and 20 μg diazaborine ml^{-1} and samples removed at various time points, a progressive increase in enzyme activity was measured (Fig. 4b). The extent of induction was dependent on the drug concentration. At the lower diazaborine concentration the β -galactosidase activity was induced 6-fold after 4 h incubation. During incubation with 20 μg of diazaborine ml^{-1} the induction of PspA occurred earlier and the enzyme activity increased by a factor of 13. The degree of growth inhibition expressed as reduction in OD_{600} as compared to the untreated control was similar at both drug concentrations (Fig. 4a). Inhibition of an essential step in fatty acid biosynthesis, namely the enoyl-ACP reductase activity, by diazaborine, thus causes high expression of the PspA protein. However, in all experiments the induction of PspA synthesis started only 3–4 h after the addition of the drug, although we know from previous experiments that lipid synthesis is inhibited immediately after diazaborine addition (Turnowsky *et al.*, 1989).

The induction of the *pspA* promoter was specific for the diazaborine action and not due to growth inhibition *per se*.

Fig. 4. Growth inhibition by diazaborine and nalidixic acid and induction of the *pspA-lacZ* fusion. (a) Growth curves of *E. coli* MC3 incubated in Minimal Medium A at 30 $^{\circ}\text{C}$ (x), at 30 $^{\circ}\text{C}$ in the presence of 4 μg ml^{-1} (Δ) and 20 μg ml^{-1} ($\blacksquare$) diazaborine or in the presence of 40 μg ml^{-1} of nalidixic acid ($\circ$). Identical cultures were incubated at 30 $^{\circ}\text{C}$ to early exponential phase before the addition of the drugs. (b) β -Galactosidase activity produced from the *pspA* promoter in the *pspA-lacZ* construct in *E. coli* MC3 was measured at the indicated time points after addition of the drugs according to Miller (1972). Diazaborine and nalidixic acid were added to early exponential phase cultures (time zero) to final concentrations of 0 μg ml^{-1} (x), 4 μg ml^{-1} (Δ) or 20 μg ml^{-1} ($\blacksquare$) of diazaborine and 40 μg ml^{-1} of nalidixic acid ($\circ$). The incubation continued for 4 h at 30 $^{\circ}\text{C}$.

Nalidixic acid at a concentration of 40 μg ml^{-1} halted growth, but did not induce the *pspA* expression (Fig. 4a, b). This result is in accordance with published data (Brissette *et al.*, 1990).

Induction of PspA by cerulenin

Cerulenin is a known inhibitor of 3-keto-acyl-ACP synthetase (D'Agnolo *et al.*, 1973). As a consequence of the inhibition of fatty acid biosynthesis, phospholipid synthesis was reduced (Fig. 5). The incorporation of orthophosphate into phospholipids after 2 h incubation in the presence of 100 μg cerulenin ml^{-1} was threefold lower than that measured in the absence of the drug. At the lower concentration, inhibition started later and was less pronounced. The effect of cerulenin on the growth of *E. coli* K12 5K is also shown in Fig. 5. At 50 μg ml^{-1} growth was only slightly affected after 3 h incubation with the

Fig. 5. Inhibition of growth and phospholipid synthesis in *E. coli* K12 5K by cerulenin. *E. coli* K12 5K was grown in phosphate-free medium at 30 °C. Identical cultures were grown to early exponential phase and 0 (×), 50 (○) and 100 µg ml⁻¹ (●) of cerulenin were added (time zero). The incubation was continued for 3 h and the OD₆₀₀ measured at the times indicated. Below the growth curves the incorporation of orthophosphate into phospholipids is shown. Cultures of *E. coli* K12 5K were grown as above. At OD₆₀₀ 0.3 (time zero) 10 µCi (370 kBq) [³²P]orthophosphate were added to each culture and incubation continued for 3 h without antibiotic (open bars), in the presence of 50 µg ml⁻¹ (grey bars) or 100 µg ml⁻¹ (black bars) cerulenin. Samples were removed at 40, 80, 120, and 160 min after the simultaneous addition of radioisotope and drug. Phospholipids were extracted as described in Methods. The radioactivity contained in aliquots of the chloroform phases was determined.

Fig. 6. Dose-dependent induction of the *pspA* promoter by cerulenin. Cerulenin was added at time zero to final concentrations of 50 µg ml⁻¹ (○) and 100 µg ml⁻¹ (●) to *E. coli* MC3 cultures grown in Minimal Medium A at 30 °C to early exponential phase and incubation continued for 6 h. Samples were removed at the indicated time points and the β-galactosidase activity determined as described. As a control, an identical culture without drug was tested (×).

drug as compared to the untreated control culture. At 100 µg cerulenin ml⁻¹, growth was strongly inhibited upon prolonged incubation without lysis of the cells.

Fig. 6 indicates the effect on β-galactosidase activity expressed by *E. coli* MC3 in response to incubation with

Fig. 7. Induction of *pspA* promoter by globomycin. Globomycin was added to early exponential phase cultures of *E. coli* MC3 (time zero) to final concentrations of 10 µg ml⁻¹ (□) and 50 µg ml⁻¹ (■) and incubation continued for 6 h at 30 °C. An untreated culture (×) served as a control.

50 and 100 µg cerulenin ml⁻¹. Cerulenin treatment at 50 µg ml⁻¹ only slightly induced PspA synthesis; the reporter enzyme activity increased 3–6-fold during 6 h incubation. However, at 100 µg ml⁻¹ we observed a strong induction of transcriptional activity from the *pspA* promoter that reached 17 times the uninduced level. The degree of induction of PspA synthesis was dependent on inhibition of growth, inhibition of phospholipid biosynthesis and correlated with the concentration of the drug. Again, the onset of induction was delayed as already observed after diazaborine treatment. Thus, the kinetics of induction by both cerulenin and diazaborine are almost identical.

Induction of PspA expression by globomycin

In order to examine PspA expression under conditions which block translocation of certain proteins through the cytoplasmic membrane we tested globomycin as a potential inducer. Globomycin is an inhibitor of the *E. coli* signal peptidase II which is specific for the processing of prolipoproteins (Inukai *et al.*, 1978a, b). When *E. coli* MC3 was incubated with 50 µg globomycin ml⁻¹ cell growth was inhibited and transcriptional activity from the *pspA* promoter was strongly induced, up to 20 times above the basal level during 6 h incubation (Fig. 7). At the lower concentration of 10 µg globomycin ml⁻¹ the induction of PspA expression was only threefold above the untreated control.

Other conditions of PspA induction

Brissette *et al.* (1990) showed that the expression of the PspA protein was induced by extreme stress conditions and by infection of *E. coli* with the filamentous phage f1. In order to test the inducibility of the *pspA-lacZ* fusion, *E. coli* MC3 R1-16 was infected with the male-specific filamentous phage M13. The β-galactosidase activity expressed by this strain was measured at various times after infection. As shown in Fig. 8 the enzyme activity

Fig. 8. Influence of phage M13 infection on *pspA* expression. The β -galactosidase activity expressed by *E. coli* MC3 R1-16 was determined as described. Phage M13 was added at a multiplicity of 10 to an early exponential phase culture of OD₆₀₀ 0.3 (time zero) and the incubation continued for 6 h at 30 °C (◆). As a control an identical culture of *E. coli* MC3 R1-16 without phage was used (×).

increased during infection with phage M13. The maximum was reached at 3 h of infection, at which time the enzyme activity was approximately 10 times higher than in the uninfected control. During longer incubation with the phage, expression of the *pspA* gene decreased slowly but stayed well above the uninduced control level.

We also tested whether the induction of PspA was mediated by alarmones. These compounds are known to accumulate upon incubation of *E. coli* cells at high temperature, in the presence of ethanol, or when incubated with oxidizing agents, like hydrogen peroxide or naphthochinon (Lee *et al.*, 1983; Bochner *et al.*, 1984). Over an incubation period of 4 h in the presence of 70 μ g hydrogen peroxide ml⁻¹ or 100 μ g naphthochinon ml⁻¹ we did not observe any induction of β -galactosidase in *E. coli* MC3 (data not shown). Thus, the effect of ethanol and high temperature on PspA synthesis is not mediated by alarmones.

Cell morphology

As described previously, treatment of *E. coli* cells with diazaborine results in a detachment of the cytoplasmic membrane predominantly at the cell poles (Turnowsky *et al.*, 1989). We observed exactly the same morphological changes when cells were treated with cerulenin at concentrations that severely inhibited phospholipid biosynthesis (data not shown). Interestingly, globomycin treatment also induces the retraction of the cytoplasmic membrane at the poles of *E. coli* cells (data not shown), although the antibiotic has a completely different mode of action and leads to the formation of large spheroplasts (Inukai *et al.*, 1978a).

DISCUSSION

We have identified the 28 kDa protein that is strongly expressed during incubation of *E. coli* with diazaborine (Turnowsky *et al.*, 1989). It is identical to the recently described phage shock protein PspA (Brissette *et al.*,

1991). Based on P1 transduction data of Brissette *et al.* (1991) we localized the *pspA* gene on the recombinant phage λ 1C2 and assigned its exact position on the *E. coli* chromosome to coordinate 1384 kb of the Kohara *et al.* (1987) physical map. It is thus closely linked to the *fabI* gene which maps at 1366.8 kb (Bergler *et al.*, 1992).

The PspA protein is associated with the cytoplasmic membrane and its synthesis is induced during infection with filamentous phages or when *E. coli* undergoes extreme stress conditions, such as incubation at 50 °C or in the presence of 10% ethanol (Brissette *et al.*, 1990). Similar incubation conditions are known to induce alarmones (Lee *et al.*, 1983). Our results, however, show that those alarmones which accumulate upon treatment with hydrogen peroxide and naphthochinon are not related with PspA synthesis. Since incubation at high temperature or in the presence of ethanol leads to the accumulation of the same alarmones as treatment with the oxidizing agents, the induction of PspA expression does not seem to proceed via alarmones.

Globomycin is an antibiotic that specifically inhibits signal peptidase II and thus leads to the accumulation of precursor forms of lipoproteins in the cytoplasmic membrane (Ichihara *et al.*, 1981). We now show that incubation of *E. coli* cells with globomycin strongly induces PspA expression. Induction of PspA was recently demonstrated for mutations affecting protein translocation (Kleerebezem & Tommassen, 1993; Carlson & Silhavy, 1993) and when large amounts of phage protein IV and other homologous proteins were integrated into the outer and cytoplasmic membranes of *E. coli* (Brissette *et al.*, 1990; Russel & Kazmierczak, 1993). Carlson & Silhavy (1993) have proposed that processing-defective outer-membrane proteins, like LamBA23D, span both inner and outer membranes and, when expressed in large amounts, affect the integrity of the envelope. As a consequence PspA synthesis is strongly induced. A truncated form of the mutant LamBA23D protein and an unprocessed form of the periplasmic MalE protein are tethered only in the inner membrane and thus fail to induce PspA synthesis. On the other hand, Kleerebezem & Tommassen (1993) suggest that accumulation of precursors within the membrane translocation complex is sufficient to induce PspA synthesis. Although these two hypotheses exist in the literature we favour the model that accumulation of unprocessed prolipoproteins after incubation of *E. coli* with globomycin could disrupt the integrity of the envelope analogous to LamBA23D.

Diazaborine treatment of *E. coli* is another novel and specific cause for induction of PspA expression. Diazaborine inhibits fatty acid biosynthesis by interfering with the NADH-dependent enoyl-ACP reductase of *E. coli* (Bergler *et al.*, 1994). Another inhibitor of fatty acid synthesis is cerulenin. A block in fatty acid biosynthesis by either antibiotic leads to inhibition of phospholipid synthesis and to strong induction of PspA. The increase in *pspA* gene expression correlates well with the degree of inhibition of phospholipid synthesis by both diazaborine and cerulenin, although the onset of induction is delayed. Inhibition of fatty acid biosynthesis certainly leads to

changes in membrane integrity which we have demonstrated microscopically as retraction of the cytoplasmic membrane after treatment of *E. coli* with diazaborine (Turnowsky *et al.*, 1989). Impaired phospholipid biosynthesis is a consequence of the block in fatty acid biosynthesis and might directly interfere with protein translocation and, thus, induce PspA synthesis or, more generally, causes severe membrane perturbations that might trigger a so far unidentified signal for subsequent induction of the PspA protein. This signal could be common also to other stress conditions that have already been known to induce PspA synthesis. Further experiments are needed to distinguish between these two alternatives.

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: John Wiley.
- Bergler, H., Högenauer, G. & Turnowsky, F. (1992). Sequences of the *envM* gene and of two mutated alleles in *Escherichia coli*. *J Gen Microbiol* **138**, 2093–2100.
- Bergler, H., Wallner, P., Ebeling, A., Leitinger, B., Fuchsbichler, S., Turnowsky, F. & Högenauer, G. (1994). Protein EnvM is the NADH dependent enoyl-ACP reductase (FabI) of *Escherichia coli*. *J Biol Chem* **269**, 5495–5496.
- Bochner, B. R., Lee, P. C., Wilson, S. W., Cutler, C. W. & Ames, B. N. (1984). AppppA and related adenylated nucleotides are synthesized as a consequence of oxidation stress. *Cell* **37**, 225–232.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Brissette, J. L., Russel, M., Weiner, L. & Model, P. (1990). Phage shock protein, a stress protein of *Escherichia coli*. *Proc Natl Acad Sci USA* **87**, 862–866.
- Brissette, J. L., Weiner, L., Ripmaster, T. L. & Model, P. (1991). Characterization and sequence of the *Escherichia coli* stress-induced *psp* operon. *J Mol Biol* **220**, 35–48.
- Carlson, J. H. & Silhavy, T. J. (1993). Signal sequence processing is required for the assembly of LamB trimers in the outer membrane of *Escherichia coli*. *J Bacteriol* **175**, 3327–3334.
- Casabadan, M. J. & Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and modification of DNA in *Escherichia coli*. *J Mol Biol* **138**, 179–207.
- D'Agnolo, G., Rosenfeld, I. S., Awaya, J., Omura, S. & Vagelos, P. R. (1973). Inhibition of fatty acid synthesis by the antibiotic cerulenin. Specific inactivation of β -ketoacyl-acyl carrier protein synthetase. *Biochim Biophys Acta* **326**, 155–166.
- Egan, A. F. & Russell, R. R. B. (1973). Conditional mutants affecting the cell envelope of *E. coli* K12. *Genet Res* **21**, 139–152.
- Garen, A. & Levinthal, C. (1960). A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochim Biophys Acta* **38**, 470–483.
- Grassberger, M. A., Turnowsky, F. & Hildebrand, J. (1984). Preparation and antibacterial activities of the 1,2,3-diazaborine derivatives and analogues. *J Med Chem* **27**, 947–953.
- Högenauer, G. & Woisetschlager, M. (1981). A diazaborine derivative inhibits lipopolysaccharide biosynthesis. *Nature* **293**, 662–664.
- Ichihara, S., Hussain, M. & Mizushima, S. (1981). Characterization of new membrane lipoproteins and their precursors of *Escherichia coli*. *J Biol Chem* **256**, 3125–3129.
- Inukai, M., Nakajima, M., Osawa, M., Haneishi, T. & Arai, M. (1978a). Globomycin, a new peptide antibiotic with spheroplast-forming activity. II. Isolation and physico-chemical and biological characterization. *J Antibiot* **31**, 421–425.
- Inukai, M., Takeuchi, M., Shimizu, K. & Arai, M. (1978b). Mechanism of action of globomycin. *J Antibiot* **31**, 1203–1205.
- Kleerebezem, M. & Tommassen, J. (1993). Expression of the *pspA* gene stimulates efficient protein export in *Escherichia coli*. *Mol Microbiol* **7**, 947–956.
- Kohara, Y., Akiyama, K. & Isono, K. (1987). The physical map of the whole *E. coli* chromosome. Application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–508.
- Lee, P. C., Bochner, B. R. & Ames, B. N. (1983). Diadenosine 5', 5'''-P¹, P⁴-tetrakisphosphate and related adenylated nucleotides in *Salmonella typhimurium*. *J Biol Chem* **258**, 6827–6834.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Russel, M. & Kazmierczak, B. (1993). Analysis of the structure and subcellular location of filamentous phage pIV. *J Bacteriol* **175**, 3998–4007.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Silhavy, T. J., Berman, M. L. & Enquist, L. W. (1984). *Experiments with Gene Fusions*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85–96.
- Turnowsky, F., Fuchs, K., Jeschek, C. & Högenauer, G. (1989). *envM* genes of *Salmonella typhimurium* and *Escherichia coli*. *J Bacteriol* **171**, 6555–6565.
- Vieira, J. & Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods Enzymol* **153**, 3–11.
- Weiner, L., Brissette, J. L. & Model, P. (1991). Stress-induced expression of the *Escherichia coli* phage shock protein operon is dependent on σ^{54} and modulated by positive and negative feedback mechanisms. *Genes Dev* **5**, 1912–1923.

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