The formation of cyclopropane fatty acids (CFAs) in *Salmonella enterica* serovar Typhimurium

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The formation of cyclopropane fatty acid (CFA) and its role in the acid shock response in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) was investigated. Data obtained by GC/MS demonstrated that the CFA level in *S. typhimurium* increased upon its entry to the stationary phase, as in other bacteria. The *cfa* gene encoding CFA synthase was cloned, and mutants of the *cfa* gene were constructed by allelic exchange. A *cfa* mutant could not produce CFA and was sensitive to low pH. Introduction of a functional *cfa* gene into a *cfa* mutant cell made the mutant convert all unsaturated fatty acids to CFAs and partially restored resistance to low pH. Interestingly, the alternative sigma factor RpoS, which was induced during the stationary phase, affected the production of C19 CFA but not C17 CFA. Western blotting analysis showed that the increase in expression of CFA synthase at early stationary phase was due to the alternative sigma factor RpoS.

**INTRODUCTION**

*Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is a neutralophilic, facultative intracellular pathogen. *S. typhimurium* is able to invade and survive within eukaryotic cells as well as to grow in various extracellular spaces. *S. typhimurium* encounters various environmental stresses, such as nutritional starvation, pH extremes, oxidative stress, osmotic shock and heat shock (Foster, 1999). *S. typhimurium* employs several strategies to avoid or tolerate acid stresses (Foster, 1999; Foster & Moreno, 1999). This adaptive process is termed the acid tolerance response (ATR). ATR is classified based on the growth phase: the exponential-phase and the stationary-phase ATR. The stationary-phase ATR has been extensively studied. The stationary-phase ATR, on the other hand, has not been characterized except for the finding that it is regulated by OmpR (Bang et al., 2000). The adaptive process is present in both exponential-phase and stationary-phase bacteria. However, stationary-phase cells, even without acid adaptation, are more tolerant to acid than exponential-phase cells. This acid resistance is not induced by low pH: it is a part of the general stress resistance induced by the stationary phase that requires the alternative sigma factor, RpoS (Lee et al., 1994).

Chang & Cronan (1999) suggested that the formation of cyclopropane fatty acids (CFAs) in the membrane is a major factor that protects *Escherichia coli* from acid shock. They also demonstrated that the sensitivity to acid shock is dependent on CFAs themselves because *cfa* mutant strains became resistant to acid shock by incorporation of CFAs from the growth medium or by introduction of a functional *cfa* gene on a plasmid.

Since the discovery of lactobacillic acid in 1950, CFAs have been detected in membrane phospholipids of a variety of eubacteria (Goldfine, 1972). These unusual fatty acids are formed in situ by the transfer of a methyl group from S-adenosyl-L-methionine to a double bond of unsaturated fatty acids (UFAs) of a phospholipid molecule (Law, 1971; Huang et al., 2002). CFA synthase, which catalyses this reaction, is encoded by the *cfa* gene (Grogan & Cronan, 1984, 1986; Taylor & Cronan, 1976). CFA synthase binds to the bilayer of the phospholipid substrate and cyclopropa-nates the phospholipid (Taylor & Cronan, 1979). This unique membrane modification occurs preferentially in the late exponential and early stationary phase (Cronan, 1968; Cronan et al., 1979; Law, 1971). In *E. coli* the *cfa* gene has been shown to have two promoters (Wang & Cronan, 1994): one promoter has the consensus sequence of a...
s^70-dependent promoter whereas the other is growth phase dependent and is recognized by the alternative sigma factor RpoS (also called s^38 and s^S). Wang & Cronan (1994) suggested that the CFA synthesis during the stationary phase is due to the increased transcription of cfa mediated by the RpoS-dependent promoter in E. coli. The s^70-dependent promoter is responsible for the low level of CFA synthesis in the exponentially growing cultures. It was also suggested that the growth-phase-dependent synthesis of CFA in E. coli was due to the instability of CFA synthase (Chang et al., 2000; Wang & Cronan, 1994). Chang & Cronan (1999) demonstrated that CFA formation could be considered as a conditional and post-synthetic modification of the bacterial membrane.

It has been reported that Salmonella spp. have CFAs in the cell membrane (Grogan & Cronan, 1997; Lechivalier, 1977; Saha & Chakraborty, 1992); however, little is known about CFA in S. typhimurium, except for its presence in the membrane. The aim of this study was to determine the pattern of CFA synthesis, the growth phase dependence of the expression of CFA synthase, and the role of CFA in the response to acid shock during stationary phase in S. typhimurium.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and their genotypes are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or minimal E medium containing 0.4% glucose (EG medium) (Vogel & Bonner, 1956). If needed, ampicillin, chloramphenicol and tetracycline were added to the media at final concentrations of 50, 30 and 20 μg/ml, respectively.

**General molecular techniques.** DNA cloning was done as described by Sambrook et al. (1989). PCR amplification was carried out with 30 cycles of amplification steps, each consisting of 30 s at

### Table 1. Strains and plasmids

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<thead>
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<th>Strain or plasmid</th>
<th>Relevant genotype or properties</th>
<th>Reference or source</th>
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<tr>
<td><strong>S. enterica serovar Typhimurium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF530 (2761)</td>
<td>Wild-type (UK-1)</td>
<td>Curtiss et al. (1991)</td>
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<tr>
<td>SF586 (JR501)</td>
<td>hsdSA29 hsdSB121 hsdL6 metE551 trpC2 ilv452 rpl120 galE719 H1-6 H2-c, n, x nml fla-66</td>
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<td>Lee et al. (1995)</td>
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<td>Bang et al. (2000)</td>
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<td>This study</td>
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<tr>
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<td>UK1 containing pACF, Tc^</td>
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<td>Gibco-BRL</td>
</tr>
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</tr>
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<td>Strain for conjugation, (−)DAP</td>
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<td>ompT hsdS dcm^, Tc^, gal(DE3) endA</td>
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<td>Cloning vector, Cm^, Tc^</td>
<td>Chang &amp; Cohen (1978)</td>
</tr>
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<td>PCR cloning vector, Ap^</td>
<td>Promega</td>
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<tr>
<td>pBlueskript(+)</td>
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<td>Edwards et al. (1998)</td>
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<tr>
<td>pECFA</td>
<td>pGEX-KG containing cfa gene, Ap^</td>
<td>This study</td>
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*DAP, DL-α,β-diaminopimelic acid.

†Δcfa, cfa gene mutated by deletion (see Methods).
Cyclopropane fatty acids in Salmonella

95 °C, 30 s at 51 °C and 1 min at 72 °C. The restriction enzymes, T4 ligase and Taq polymerase were purchased from Roche, Takara and GENEMED, respectively. General transduction was performed with P22 HT105/int, and nonlysogenic segregants were identified by sensitivity to P22 H5 (Maloy, 1990).

Mutation of the CFA synthase gene (cfa). In order to delete part of the cfa gene (deletion fragment, 870 bp), fusions of DNA fragments were generated by PCR as follows. To clone the upstream part of the cfa gene, the primers KCFAU1 (5'-ggacttCACGAGCGTTAATCATGTTAATAGG-3') and KCFAD2 (5'-tttaggcGCGGAAATACCACCTG-3') were used for amplifying the downstream fragment; the 5' tails, which contained cleavage sites for SacI and BanHI (underlined) are shown in lower case. The primers KCFAD1 (5'-ggacttTTGCAGGAAATCACGTTAATAGG-3') and KCFAD2 (5'-tttaggcGCGGAAATACCACCTG-3') were used for amplifying the downstream fragment; the 5' tails, which contained cleavage sites for SacI and BglII (underlined). The PCR products were cloned into pGEM T-easy vector (Promega) and the cloned vectors were digested with restriction enzymes as described above. Subsequently, the digested upstream and downstream fragments of cfa gene were ligated by T4 ligase (Takara). Using the product of this ligation as a template, a further PCR was carried out with the primers KCFAU1 and KCFAD2; the product was cloned into pGEM T-easy vector (forming pTDCF).

In order to insert a drug-resistance marker (ampicillin-resistance gene, bla) between the upstream and downstream fragments, the ampicillin-resistance gene derived from the pGEM T-easy vector was amplified by PCR with the synthetic oligonucleotide primers KCFAU1 (5'-ggacttATGCGGGGAGATGATTA-3') and amp2 (5'-ggacttacaCTACGTTAAGGGATTTTGTGAATG-3'), which carry 5' tails (lower case) and a cleavage site for BanHI (underlined). The PCR product was digested with BanHI and cloned into pTDCF (forming pTAPC). Plasmids pTDCF and pTAPC were digested with SacI and XbaI, and each fragment was cloned into the suicide plasmid pDMS197, which contains the counter-selectable sacB marker. The resulting clones, named pDCF and pAPC, respectively, were transformed into E. coli 7215, and the transformants were conjugated with wild-type S. typhimurium (Edwards et al., 1998; Fu & Voordouw, 1997). The cfa deletion mutants and bla insertion mutants were selected in a medium containing 5% (w/v) sucrose and ampicillin, as described by Edwards et al. (1998). The acid tolerance response (ATR) assay. The acid-induced stationary-phase ATR was measured as described by Bang et al. (2000). Briefly, cells were grown in 3 ml minimal E medium (pH 8.0; 37 °C; shaking) overnight for 16 h. Then 500 μl sample of each strain was harvested by centrifugation and washed in an equal volume of EG broth at pH 8.0 for the unadapted culture. The samples were reharvested, and the pellets were resuspended in 3 ml EG broth at pH 3.0 for challenge. Aliquots were collected at timed intervals, and viable counts were measured by serial dilution and plating on LB agar. The results are representative of triplicate experiments, with variability observed within 50% of the reported value.

Cloning of a functional cfa gene and complementation. To clone the functional cfa gene, the cfa structural gene plus its upstream region was amplified by PCR with the synthetic oligonucleotide primers CCFAA-L (5'-ccgacctgGACCACCAACCGGTATA-3') and CCFAA-R (5'-ccgacctgGACCGACCAGCATGTTTGTG-3'), which contain cleavage sites for BanHI and HindIII (underlined) within their 5' tails (lower case). The template for PCR was obtained from the wild-type strain UK-1. The PCR product was digested with BanHI and HindIII and cloned into pACYC184, to produce pACF, containing the functional cfa gene.

After culturing overnight, wild-type, rpoS and cfa mutants of S. typhimurium were inoculated into 50 ml fresh LB medium and incubated at 37 °C with vigorous shaking until the OD600 reached 0.5. Cells were chilled in ice, centrifuged, and the pellets were washed twice with ice-cold glycerol/water, and resuspended in a volume of 15% glycerol/water equal to that of the pellet. Samples of cells (50 μl) were electroporated with 10 ng pACF by using a Gene Pulser II Electroporation system (Bio-Rad). The transformants were tested for their acid shock sensitivity and fatty acid formation phenotype.

Purification of CFA synthase. The CFA synthase structure gene cfa was amplified by PCR with the synthetic oligonucleotide primers ECF-A-L (5'-aagaattCTAGAGTTTATCATGTTAATAGG-3') and ECF-A-R (5'-aagaattATGCGGGGAGATGATTA-3'); the 5' tails (lower case) contained cleavage sites for BanHI and HindIII (underlined). The PCR product was digested with BanHI and HindIII and cloned into pGEX-KG vector (forming pECFA). The CFA synthase–GST fusion protein was purified according to the manufacturer's manual (Amersham Pharmacia Biotech). Glutathione-Sepharose 4B resin (Amersham) was added to the solution containing the purified fusion protein, mixed gently for 30 min at room temperature, and the mixture was centrifuged at 500 g for 3 min. After washing the resin four times with 15 ml PBS buffer to remove unbound proteins, CFA synthase was eluted with 20 mM Tris/HCl (pH 8.4) containing 0.04 U thrombin, 50 mM NaCl and 2.5 mM CaCl2 at 22 °C for 22 h. The CFA synthase thus obtained was further purified by preparative SDS-PAGE. The fractions containing CFA synthase were pooled and dialysed against 0.1 M potassium phosphate buffer (pH 7.5).

Antibody preparation. Using the CFA synthase purified as described above, antiserum was prepared by injecting rabbits with 150 μg of the protein dispersed in complete Freund's adjuvant. Two weeks and four weeks later, the rabbits were immunized again with 50 μg CFA synthase dispersed in incomplete Freund's adjuvant.

Western blotting. The CFA synthase protein was detected by immunoblot analysis as described previously (Bang et al., 2000), with the following modifications. Western blots were treated with a 1:1000 dilution of polyclonal antibody against CFA synthase, followed by incubation with an alkaline-phosphatase-labelled anti-rabbit antibody. Blots were developed by using a chemiluminescent detection kit. To enhance specificity, the antiserum was incubated with proteins from the cfa mutant S. typhimurium strain at 4 °C. The data were obtained from three independent experiments.

Preparation of total fatty acids. To prepare the total fatty acids from stationary-phase cells, cultures were grown overnight for 16 h. To obtain cells in the exponential growth phase, overnight-cultured cells were inoculated by 1:100 dilution to fresh LB medium and were cultured until the OD600 reached 0.6. Cells thus obtained were centrifuged for 20 min at 5000 r.p.m. The pellet was washed twice with distilled water. The washed pellets were frozen at −20 °C, and then freeze-dried with a vacuum freeze drier.

The total fatty acids extract of cells was prepared as described by Moss & Dees (1975) with the following modifications. The lyophilized cells were saponified by hydrolysis with 10% (w/v) sodium hydroxide in 50% (v/v) methanol. The saponified solution was acidified with 14% boron trifluoride in 50% (v/v) methanol. Fatty acid methyl esters were extracted using n-hexane/dichloromethane. The upper phase of the washed extract was analysed by GC/MS using an HP5890 Series II gas chromatograph combined with an HP5970B mass spectrometer (Hewlett Packard).

Analysis of fatty acid methyl esters by GC/MS. Fatty acid methyl derivatives were analysed by GC/MS at 70 eV in scan mode, using an HP-5MS capillary column (25 m×0.20 mm i.d., with a film thickness of 0.33 μm). The GC injection port temperature was 250 °C and the transfer line temperature was 300 °C. The split ratio was 1:10. Helium was used as a carrier gas (flow rate 0.8 ml
min\(^{-1}\)). The operating conditions were as follows: an initial oven temperature of 150 °C was held isothermal for 4 min; a temperature programme of 5 °C min\(^{-1}\) continued to a final oven temperature of 300 °C, which was held isothermal for 11 min in order to remove contaminants. The total run time was 45 min. The fatty acids were identified by comparison of retention times and mass fragmentation patterns with authentic standards purchased from Supelco.

RESULTS

CFA synthase from S. typhimurium

Before complete genome sequencing of Salmonella enterica serovar Typhimurium (S. typhimurium) revealed that a putative cfa synthase gene was present in its genome (McClelland et al., 2001), we had already sequenced a putative cfa gene from S. typhimurium (accession no. AF417203). The gene had 87% nucleotide identity with the cfa gene, and the deduced amino acid sequence showed 90% identity and 95% similarity with cyclopropane fatty acid (CFA) synthase from E. coli (data not shown). It is not known whether CFA synthase from S. typhimurium shares common properties with other S-adenosyl-L-methionine (S-AdoMet)-utilizing enzymes such as E. coli CFA synthase. S-AdoMet-utilizing enzymes usually have three amino acid sequence motifs (DxxGxG, GxGG and VLDxGxGxG) (Wang et al., 1992). The CFA synthase from S. typhimurium was compared with other S-AdoMet-utilizing enzymes containing such motifs, and was found to contain each of the motifs within the sequence VLDIGCGWGG (residues 171–182). It also included the VLDxGxGxG sequence that is known to play a role in the S-AdoMet binding (Haydock et al., 1991; Ingrosso et al., 1989).

Formation of CFAs in S. typhimurium

To investigate the composition of membrane fatty acids during the various growth phases in S. typhimurium, the total fatty acids from exponential- and stationary-phase cells were prepared and analysed by GC/MS. During the exponential phase only low levels of C17 CFAs were detected (Fig. 1a). In stationary-phase cultures the level of C17 CFAs increased and C19 CFAs were also present (Fig. 1b). However, the content of saturated fatty acids in the membrane increased only slightly and the content of hydroxy fatty acids was constant regardless of the growth phase (Fig. 1c). The total CFA level began to increase between mid-exponential phase and the stationary phase (OD\(_{600}\) 0.6–0.9) (Fig. 1c). Fig. 1(c) also shows a dramatically altered composition of fatty acids: unsaturated fatty acids (UFAs) were nearly quantitatively replaced by their cyclopropane derivatives during stationary phase: methyl cis-9-hexadecanoate (C16:1\(^\Delta 9\)) and methyl cis-11-octadecanoate (C18:1\(^\Delta 11\)) were replaced by methyl cis-11,12-methylenehexadecanoate (C17:0\(^\Delta 5\)) and methyl cis-9,10-methyleneoctadecanoate (C19:0\(^\Delta 4\)), respectively.

A cfa mutant is unable to produce CFAs

Using the suicide vector (pDMS197, a gift from R. Curtiss, Seattle, WA, USA) on the basis of allelic exchange, cfa mutants were constructed as described in Methods. A functional ampicillin-resistance gene (bla) was used to replace the cfa locus on the chromosome (Fig. 2a). The mutation of cfa was confirmed by PCR (Fig. 2b). The cfa insertion mutation does not exert a polar effect on the downstream genes because cfa is expressed as a monocistronic mRNA and the downstream gene (ribE: riboflavin synthase) is
divergently transcribed (see Fig. 2a). To examine the ability of a cfa mutant to produce CFA, the total fatty acids from stationary-phase cells were extracted and analysed by GC/MS. Fig. 2(c) indicates that the cfa mutant containing a functional ampicillin-resistance gene (bla) was unable to synthesize CFA. The cfa deletion mutant was also unable to synthesize CFA (data not shown). We examined whether the cfa gene was responsible for the formation of CFA in S. typhimurium by introducing the low-copy-number plasmid pACF, which carries a functional cfa gene, into the mutant. The functional cfa gene contains the putative promoter region (260 bp from the start codon) and the structural gene. The total fatty acids from the cfa mutant containing pACF at stationary phase were analysed by GC/MS. As shown in Fig. 2(d), all UFA was converted to CFA at stationary phase. The complete conversion of UFA to CFA appears to be the result of the overexpression of CFA synthase. The overexpression did not affect the growth of S. typhimurium (data not shown). The results indicate that the putative cfa gene is responsible for the conversion of UFA to CFA in S. typhimurium.

RpoS regulates the formation of C19 CFAs during stationary phase

A cfa mutant was unable to produce CFAs (Fig. 2c), which indicates that CFA synthase increases the production of C17 and C19 CFAs in the stationary phase. In E. coli, it has been suggested that the initiation of CFA synthesis at the entry to the stationary phase is due to the increased transcription of cfa by the RpoS-dependent promoter (P2) whereas the σ70-dependent promoter (P1) is responsible for the low level of CFA synthesis in exponentially growing cultures (Eichel et al., 1999; Wang & Cronan, 1994). We therefore compared the promoter regions of the CFA synthase genes of S. typhimurium and E. coli. Fig. 3 shows that the promoter region of S. typhimurium is highly homologous to the two promoters (P1 and P2 regions) of the cfa gene in E. coli. We thus speculated that RpoS from S. typhimurium might increase the CFA synthase level during the stationary phase, and examined the effect of RpoS on the formation of CFAs in S. typhimurium. The total CFA content of the wild-type and rpoS mutant was compared in both the exponential and stationary growth
In the exponential phase, the level of C17 and C19 CFAs was comparable between the wild-type and the \textit{rpoS} mutant (Table 2). In the stationary phase, on the other hand, the \textit{rpoS} mutant did not produce C19 CFAs whereas the wild-type cells produced C19 CFAs normally (Table 2). The level of C17 CFAs increased in the \textit{rpoS} mutant during stationary phase to a level comparable with the wild-type (Table 2). The results indicated that RpoS increased the formation of C19 CFAs but not C17 CFAs in the stationary phase. In \textit{E. coli}, on the other hand, it was reported that less than 50 $\%$ of C16 UFAs and 10 $\%$ of C18 UFAs were converted to the corresponding CFAs in an \textit{rpoS} mutant (Wang & Cronan, 1994). The latter study suggested that this phenomenon was due to the reduced level of CFA synthase, caused by the deficiency of RpoS in the stationary phase. We thus examined whether the overexpression of CFA synthase in an \textit{rpoS} mutant could restore its ability to produce C19 CFA normally. Hence, we measured the level of C19 CFAs in \textit{rpoS} mutant and wild-type strains containing pACF in both exponential and stationary phases (Table 2). The overexpression of CFA synthase made both the mutant and the wild-type produce C19 CFA even in the exponential phase. This indicates that the CFA synthase level is essential to the formation of CFA in \textit{S. typhimurium}. In the exponential phase, the C19 CFA level in the wild-type containing pACF was similar to that in the \textit{rpoS} mutant cells containing pACF (Table 2). Also, the C17 CFA level in the wild-type containing pACF was similar to that in the \textit{rpoS} mutant containing pACF (Table 2). In the stationary phase, however, the C19 CFA level in the \textit{rpoS} mutant containing pACF did not reach the wild-type level (Table 2). Interestingly, the \textit{rpoS} mutant containing pACF produced more C17 CFA than the wild-type containing pACF in the stationary phase (Table 2).

### Expression of CFA synthase increases at the onset of stationary phase

As the discrepancy of C19 CFA level between two strains in the stationary phase could be the result of their different level of expression of CFA synthase, we examined the expression level of the enzyme by Western blotting using polyclonal anti-CFA synthase antibody, prepared as described in Methods. However, this antibody bound to numerous other proteins, impeding the detection of CFA synthase from the wild-type. This difficulty may be due to

**Table 2. Content of fatty acids in the cell membrane**

The compositions were determined by GC/MS of cellular fatty acid methyl esters (see Methods). \textit{rpoS} and \textit{rpoS/pACF} indicate strains JF2938 and YK4042, respectively (see Table 1). CFA content is given as the relative peak area [(peak area of CFA or UFA/total peak area) $\times$ 100 $\%$]; the data are means of triplicate experiments. The compositions of CFA are shown in bold.

<table>
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<tr>
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<th>C16 UFA</th>
<th>C17 CFA</th>
<th>C18 UFA</th>
<th>C19 CFA</th>
<th>C16 UFA</th>
<th>C17 CFA</th>
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</table>

**Fig. 3.** Comparison of the promoter regions of the \textit{E. coli} (Ec) and the \textit{S. typhimurium} (St) \textit{cfa} genes. P1 and P2 indicate the two promoter regions of the \textit{E. coli} \textit{cfa} gene (Wang & Cronan, 1994). The boxes indicate –35 and –10 sequences from P1 and P2 regions in the \textit{E. coli} \textit{cfa} promoter. The putative Shine–Dalgarno sequence is underlined. The start codons are in bold letters. The nucleotide sequence of \textit{E. coli} was obtained from Wang & Cronan (1994). The \textit{S. typhimurium} sequence was obtained from GenBank accession no. AF417203.
the low expression level of CFA synthase in addition to the nonspecific binding of antibodies. To investigate the expression level of CFA synthase, we therefore employed the wild-type and rpoS mutant harbouring pACF. Since the functional cfa gene is inserted divergently into the tetracycline-resistance gene (tetR) on pACYC184, the promoter of the tetR gene does not influence the expression of the cfa gene. During exponential phase (OD_600 0.45), the expression level of CFA synthase was similar in both the wild-type and the rpoS mutant containing pACF (Fig. 4, lanes 1, 5). On the other hand, CFA synthase expression was increased when wild-type cells started to enter stationary phase, but the expression in rpoS mutant cells was not increased (Fig. 4, lanes 2, 3, 6, 7). Therefore, in the stationary phase, the difference in the level of C19 CFAs may be due to differences in the level of expression of CFA synthase between the wild-type and the rpoS mutant. However, we have already shown that in the stationary phase, the rpoS mutant containing pACF produced more C17 CFAs than the wild-type containing pACF (Table 2), although there was a lower level of the CFA synthase in the rpoS mutant in the stationary phase (Fig. 4). From these results, it seems that another factor may be involved in the formation of C19 CFA in stationary phase.

**A CFA synthase mutant is sensitive to low pH**

It has been demonstrated that the resistance to acid shock of *E. coli* is affected by the CFA content (Brown et al., 1997; Chang & Cronan, 1999). To investigate whether the CFA content in the cell membrane of *S. typhimurium* is related to the resistance to low-pH conditions, an ATR assay in stationary phase was carried out without acid adaptation, as described in Methods. As shown in Fig. 5(a, b), the cfa mutant was more sensitive than the wild-type to acid shock.

<table>
<thead>
<tr>
<th>Wild-type/pACF</th>
<th>cfa::Ap</th>
<th>rpoS/pACF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSP</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CFA synthase</td>
<td>0-45</td>
<td>0-85</td>
</tr>
<tr>
<td>OD_600</td>
<td>0-45</td>
<td>0-85</td>
</tr>
</tbody>
</table>

**Fig. 4.** Expression of CFA synthase is increased during early stationary phase. Western blotting of CFA synthase in the wild-type strain containing pACF (lanes 1, 2, 3) and in an rpoS mutant strain containing pACF (lanes 5, 6, 7). Lane 4, ‘cfa::Ap’, indicates the absence of expression from the cfa mutant during the stationary phase, and serves as negative control. OD_600 0.45, 0.85 and 1.0 indicates the mid-exponential (lanes 1, 5), the early stationary (lanes 2, 6) and the stationary phase (lanes 3, 7), respectively. Because non-specific protein (NSP) is constitutively expressed, it can be used as a control for equal total protein loading.

To determine whether the increased acid sensitivity of the mutant is due to the CFA deficiency, plasmid pACF, which carries a functional cfa gene, was introduced into the mutant. The presence of pACF partially restored the acid resistance (Fig. 5b). We observed previously that a cfa mutant with pACF converted all UFAs to CFAs in stationary phase (Fig. 3d). This incomplete restoration of acid resistance may be due to the deficiency of UFAs in the cell membrane. However, because cells deficient in CFAs were more sensitive to acid shock than UFA-deficient cells, these data demonstrate that cells with membranes containing CFAs resist acid shock better than the corresponding UFA-containing cells.

We demonstrated that the synthesis of CFA is partly dependent on the RpoS sigma factor (Table 2). Previous data showed that the induction of RpoS in the stationary phase rendered cells resistant to general stresses such as acid shock (Lee et al., 1994; Small et al., 1994). Here, we constructed an rpoS cfa double mutant and compared its acid tolerance response with each single mutant. During stationary phase, the double mutant strain completely lacked CFAs in the membrane (data not shown), whereas...
residual CFAs remained in the rpoS mutant strain (Table 2). It was expected that strains lacking RpoS as well as CFA would be more sensitive than either single mutant strain. As shown in Fig. 5(a), the resistance of the double mutant to acid shock was indeed more impaired than that of the single mutant.

**DISCUSSION**

It has been reported that in most Gram-negative bacteria, CFAs are synthesized during the stationary phase (Cronan, 1968; Grogan & Cronan, 1997; Law, 1971). In this study, we showed that like other bacteria, *S. typhimurium* produced CFAs at the beginning of the stationary phase (Fig. 1). Also, we showed that the alternative sigma factor, RpoS, affected the production of C19 CFAs but not C17 CFAs in stationary phase (Table 2). This seems to be due to the reduced level of CFA synthase, caused by the deficiency of RpoS in the stationary phase. We thus examined whether the overexpression of CFA synthase in an rpoS mutant could restore its ability to produce C19 CFA normally. In the stationary phase, however, the C19 CFA level in the rpoS mutant containing pACF did not reach the wild-type level (Table 2). This suggests that in *S. typhimurium*, the effect of RpoS on C19 CFA formation may not be entirely due to the level of expression of CFA synthase.

In this work, we also showed that in the stationary phase, all C18 UFAs were converted to C19 CFAs in the rpoS mutant cells overexpressing CFA synthase (Fig. 2d, Table 2). In mid-exponential phase, however, all C18 UFAs were not converted to C19 CFAs in the rpoS mutant cells overexpressing CFA synthase (Table 2). The discrepancy could be due to different levels of expression of CFA synthase in the exponential and the stationary phase. Our data, however, showed that the CFA synthase level in an rpoS mutant harbouring pACF was constant regardless of the growth phase (Fig. 4, lanes 5, 6, 7). The results suggest that the distribution ratio of total CFAs in *S. typhimurium* may be determined by the specific activity of an enzyme in vivo during the stationary phase. Alternatively, another factor, which is induced by RpoS in stationary phase, may affect the distribution ratio of total CFAs. However, it is certain that the CFA synthase level is essential to the synthesis of CFAs.

In *E. coli*, the cyclization of fatty acid acyl chains is generally regarded as a means of controlling the penetration of undesirable molecules in order to adapt the cells to adverse conditions (Chang & Cronan, 1999; Grogan & Cronan, 1997). Among *Helicobacter* isolates, those identified as gastric colonizers tend to produce a large amount of CFAs. The isolates identified as intestinal colonizers, on the other hand, generally do not produce a large amount of CFAs (Haque et al., 1996). In addition, *Enterococcus faecalis* mutants resistant to folic acid antagonists were found to be CFA deficient and more sensitive to acid than the parental strain (Jungkind & Wood, 1974). Chang & Cronan (1999) suggested that the protection of cells from acid shock by CFAs might also apply to other bacteria. Here, we considered that protection against acid shock by forming CFAs could also apply to *S. typhimurium*. It is known that RpoS from *S. typhimurium* regulates other processes as well as synthesis of CFAs (Lee et al., 1994; this study). Therefore, it is thought that other physiological processes regulated by RpoS can mask the effects of CFA content on acid shock survival. In this study, however, we showed that in *S. typhimurium*, an rpoS cfa double mutant was more sensitive to acid shock than either single mutant strain, as is the case for *E. coli* (Chang & Cronan, 1999; this study, Fig. 5a). These results indicate that the residual presence of CFAs in an rpoS mutant allows cells to survive longer against acid shock, because formation of CFAs was not completely blocked in rpoS mutants of *E. coli* and *S. typhimurium* (Chang & Cronan, 1999; this study). Therefore, it is thought that the presence of CFA in the cell membrane is essential to acid tolerance in both organisms.

In this study, we also showed that complete conversion of UFAs to CFAs did not restore the stationary-phase ATR of *S. typhimurium* fully (Fig. 5b). This incomplete restoration of acid resistance may be due to the deficiency of UFAs in the cell membrane. Chang & Cronan (1999) showed that incorporation of exogenous C18 UFA into a fabA/cfa mutant strain partially restored acid resistance in *E. coli*. From these data, we suggest that UFAs as well as CFAs are necessary for full acid resistance of *S. typhimurium*, although CFAs are a better barrier against acid shock than UFAs.

It has been reported that in *S. typhimurium*, RpoS was induced by mild acidic shock during the exponential phase (Lee et al., 1994, 1995). Recently, de Jonge et al. (2003) showed that under acid-adaptive conditions, both the C17 and the C19 CFA content of *S. typhimurium* strains increased. It was thus speculated that the acid-induced RpoS in the exponential phase might increase CFA synthase activity in *S. typhimurium*. Hence, we compared the CFA level in mildly acidic conditions (pH 5-8) and normal conditions (pH 7) during the exponential phase; we observed that the exposure to the acidic condition increased the CFA level (data not shown). It has been reported that the adaptation to mild (pH 5-8) or moderate (pH 4-4) acidic conditions renders cells tolerant to severe acid stress (pH 3) (Lee et al., 1995). Therefore, it is suggested that the sustained tolerance to acid shock may be partly due to the increase of CFA level by the mild acid shock-induced RpoS during the exponential phase.

DNA base sequence variability in the rpoS genes of various wild-type strains has been reported. The variability correlated with phenotypic differences in the expression of RpoS-controlled genes (Ivanova et al., 1992). Such strain differences may explain the considerable variation in the CFA synthase level observed in several strains of *E. coli* K-12 expressing various plasmid and phage cfa clones (Grogan & Cronan, 1984). Wang & Cronan (1994) showed that *E. coli* strain FT1 had a lower CFA synthase activity
than strain ZK126. They suggested that the difference in the CFA synthase level may be the result of different ΔpoIS alleles, since the expression of another RpoS-regulated gene, poxB, was lower in FT1 than ZK126 (Wang & Cronan, 1994). Here, we used only one S. typhimurium strain for the analysis of CFA formation. To elucidate the relationship between RpoS and the formation of the CFAs in S. typhimurium more extensively, the analysis of various Salmonella strains is required.

In conclusion, we have demonstrated the formation of CFAs, the physiological role of CFAs, and the expression of CFA synthase in S. typhimurium. We believe that these findings represent the first report of the physiological significance of CFAs in S. typhimurium. To elucidate the mechanism of the increase of CFA content by CFA synthase during the stationary phase in S. typhimurium, further investigation of the transcription of the cfa gene, and the stability and activity of the CFA protein, is required. The identification of other factor(s) regulating the formation of C19 CFA may also be worthwhile.

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