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Involvement of Cyclopropane Fatty Acids in the Response of *Pseudomonas putida* KT2440 to Freeze-Drying

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***Pseudomonas putida* KT2440, a saprophytic soil bacterium that colonizes the plant root, is a suitable microorganism for the removal of pollutants and a stable host for foreign genes used in biotransformation processes. Because of its potential use in agriculture and industry, we investigated the conditions for the optimal preservation of the strain and its derivatives for long-term storage. The highest survival rates were achieved with cells that had reached the stationary phase and which had been subjected to freeze-drying in the presence of disaccharides (trehalose, maltose, and lactose) as lyoprotectants. Using fluorescence polarization techniques, we show that cell membranes of KT2440 were more rigid in the stationary phase than in the exponential phase of growth. This is consistent with the fact that cells grown in the stationary phase exhibited a higher proportion of $C_{17:cyclopropane}$ as a fatty acid than cells in the exponential phase. Mutants for the *cfaB* gene, which encodes the main $C_{17:cyclopropane}$ synthase, and for the *cfaA* gene, which encodes a minor $C_{17:cyclopropane}$ synthase, were constructed. These mutants were more sensitive to freeze-drying than wild-type cells, particularly the mutant with a knockout in the *cfaB* gene that produced less than 2% of the amount of $C_{17:cyclopropane}$ produced by the parental strain.**

Pseudomonas putida KT2440 is a well-characterized saprophytic member of the family *Pseudomonaceae* (38) certified by the National Institutes of Health as a secure host for foreign gene cloning (3). This microorganism uses a number of aromatic compounds, amino acids, and organic acids as carbon and energy sources (2, 34, 39), and it is able to adhere to abiotic and biotic surfaces, being an excellent colonizer of the plant roots (26). The complete genome sequence of *P. putida* KT2440 (27) reveals its nonpathogenic nature and also its potential for applications in agriculture, biocatalysis, bioremediation, and bioplastic production (19, 27, 30).

The availability of complete genome sequences facilitates research with genomics and proteomics, although many of these studies require a collection of mutants large enough to establish unequivocal gene and function relationships to identify regulons and to determine the hierarchy in transcriptional processes. We have constructed an ordered bank of mutants of *P. putida* (E. Duque [www.artemisa.eez.csic.es]) to facilitate detailed functional genomic studies of this microorganism. Long-term storage conservation of the mutant collection is of critical importance. Freezing at −80°C is frequently used for high-throughput analyses and to search for defined phenotypes. However, lyophilization is the most appropriate method for the long-term preservation of the clones. Lyophilization involves the use of lyoprotectants and myoinositol has been used with gram-negative bacteria in general (13, 29, 37) and with *Pseudomonas* in particular (E. Duque, unpublished results). However, upon lyophilization of *P. putida* KT2440, the recovery rate is on the order of 1 out of 10^4 original cells. To improve survival after lyophilization, we explored here the effect of growth conditions, growth phase, and the nature of lyoprotectants on the lyophilization of *P. putida* KT2440.

Our results indicated that survival of the strain was influenced by the bacterial growth phase and by the type and concentration of lyoprotectants used. We found that growth conditions that result in an increase in the relative amount of $C_{17:cyclopropane}$ fatty acid yielded better survival after lyophilization and that of the compounds we tested trehalose was the best lyoprotectant. We identified *cfaB* as the gene that encodes the major $C_{17:cyclopropane}$ synthase in KT2440 and generated a *cfaB* mutant which, in consonance with the low $C_{17:cyclopropane}$ levels, exhibited reduced survival rates after lyophilization.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in the present study are listed in Table 1. Bacterial cells were grown at 30°C in a Kühner orbital shaker operated at 200 rpm in LB medium or M9 minimal medium containing 20 mM concentrations of different carbon sources (benzoate, choline, citrate, fructose, glucose, proline, and sucrose) (26).

Lyophilization of *P. putida* KT2440. *P. putida* KT2440 cells were grown until the desired turbidity, and then bacterial cells were harvested by centrifugation (7,500 × *g* for 10 min), washed twice with phosphate buffer, and resuspended in 30 ml of a lyoprotectant solution. Before the cells were freeze-dried the number of viable cells was counted in triplicate by spreading serial dilutions in LB solid medium. Different compounds were used as lyoprotectans, i.e., amino acids, disaccharides, monosaccharides, polyalcohols, and skimmed milk (Table 2). Sterile ampoules were filled with 500 μl of the bacterial suspension containing the lyoprotectant and covered with sterile cotton placed in the middle part of the ampoule. Ampoules with the bacterial suspension were first frozen at −20°C for 20 min and then submerged in liquid nitrogen. Subsequently, the freeze-drying process was carried out in a Virtis lyophilizer at 30 mtorr for 48 h. After the process, the ampoules were sealed under vacuum. At least three ampoules were used to determine the number of viable cells after lyophilization. To this end, 500

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TABLE 1. Bacterial strains and plasmids used in this study[a]

Strain or plasmid	Relevant characteristics[b]	Source or reference
Strains		
P. putida KT2440		
Wild type	Prototrophic	27
RpoS	*rpoS*::miniTn*5*::*lux*	36
cfaA mutant	Mutant in *cfaA* inactivated by a Km cassette	This study
cfaB mutant	Mutant in *cfaB* inactivated by a Gm cassette	This study
E. coli		
DH5α	*recA*; host for cloning	18
CC118λ*pir*	Rif^r; host for pKNG101 plasmid and its derivatives	18
Plasmids		
pKNG101	Sm^r	20
pUC18Not	Ap^r; cloning vector	18
pRK600	Cm^r; helper plasmid	18
pUK18	Ap^r Km^r; source of the Km^r cassette	25
pMS265	Ap^r Gm^r; source of the Gm^r cassette	4
pKNG101cfaAKm	Sm^r Km^r	This study
pKNG101cfaBGm	Sm^r Gm^r	This study
pUcfaA	Ap^r; pUC18Not derivative bearing *cfaA*	This study
pUcfaB	Ap^r; pUC18Not derivative bearing *cfaB*	This study
pUcfaAKm	Ap^r Km^r	This study
pUcfaBGm	Ap^r Gm^r	This study

[a] All mutant strains have been deposited in the *P. putida* reference culture collection and can be obtained from E. Duque.

[b] Rif^r, rifampin resistance; Sm^r, streptomycin resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

μl of M9 buffer solution was added to cells, and after 40 min, with occasional shaking, rehydratation was achieved. Bacterial cells were serially diluted and viable cells counted on LB plates.

The bacterial survival ratio (BSR) is reported as the ratio of the log of the number of bacterial cells present in the suspension after lyophilization (AL) to the log number of viable cells before lyophilization (BL) multiplied by 100, i.e., $BSR = (\log_{AL}/\log_{BL}) \times 100$.

Analysis of fatty acid profile. *P. putida* KT2440 cells were grown for different periods of time and phospholipids were extracted by the method of Bligh and Dyer (5). To measure fatty acids, phospholipids were transesterified, and methyl ester derivatives were identified by mass spectrometry after gas chromatographic separation as described previously (35).

Membrane fluidity determinations. Membrane fluidity was determined by measuring the fluorescence polarization of the probe DPH (1,6-diphenyl-1,3,5-hexatriene) inserted into the cytoplasmic membranes. Cells in two different growth phases were harvested by centrifugation (10,000 × *g* for 1 min), washed in sterile 15 mM Tris-HCl buffer (pH 7.0), and resuspended in same buffer to a turbidity at 660 nm of 0.2. Then, 1 μl of the fluorescent membrane probe (12 mM stock solution in tetrahydrofuran) was added to 3 ml of the resuspended culture in a quartz cuvette to obtain a final concentration of 4 μM and incubated with a magnetic stirring at 200 rpm for 10 min in the dark at 30°C to allow probe incorporation into the cytoplasmic membrane (43). Fluorescence polarization was measured by using a spectrofluorimeter (Photon Technology International, Inc.) equipped with a thermostated stabilized cell cuvette holder and a cuvette stirrer. The excitation wavelength for the DPH probe was 358 nm, and the emission wavelength was 428 nm. The slit width for the excitation and emission beams was 12 and 10 nm, respectively. The intensities parallel (I_{VV}) and perpendicular (I_{VH}) to the vertically polarized excitation beam were recorded and the degree of polarization (P) calculated from the intensity measurements with the equation $P = (I_{VV} - I_{VH}G)/(I_{VV} + I_{VH}G)$, where G was the correlation factor for instrument polarization. G values were calculated as the ratio of vertical to horizontal measurements when the excitation light was polarized horizontally; the higher the value of polarization, the lower the membrane fluidity. Values were analyzed by using the FELIX (PTI) software program (43).

Construction of *cfaA*- and *cfaB*-null mutants of *P. putida* by gene replacement. The original annotation of the *P. putida* KT2440 genome identified two potential open reading frames encoding $C_{17:cyclopropane}$ synthase (PP2734 and PP5365). These enzymes have been named CFA-A and CFA-B based on our work. Mutants of *P. putida* KT2440 with knockouts in the *cfaA* and *cfaB* genes were constructed by reverse genetic procedures. All plasmids for allelic replacements were based on the pKNG101 suicide vector and are listed in Table 1. Plasmids pKNG101cfaAKm and pKNG101cfaBGm bear truncated *cfaA* and *cfaB* alleles that were marked with kanamycin (Km) and gentamicin (Gm), respectively. *Escherichia coli* CC118λ*pir* (pKNG101cfaAKm) was mated with *P. putida* KT2440 in the presence of the helper *E. coli* HB101(pRK600). *Pseudomonas putida* KT2440 transconjugants were selected on M9 minimal medium with benzoate as the carbon source and Km. The nature of the cointegration event was confirmed by PCR. A random clone was chosen and grown on LB without Sm for 20 generations. Then cells were spread on LB Km plates supplemented with 7% (wt/vol) of sucrose. Among the sucrose-resistant clones we searched for the Sm-sensitive ones as putative resolved clones. Several such clones were found, and upon confirmation of gene replacement by Southern blot a random clone was retained for further assays. This mutant strain was called KT2440-*cfaA*.

To construct KT2440 derivatives with a knockout in *cfaB*, we proceeded as described above except that we used pKNGcfaBGm to generate a chromosomal knockout in *cfaB*. A single mutant clone, KT2440-*cfaB* was retained for further work.

Statistical analysis. Data for different experiments were compared by using the Student *t* test.

RESULTS

Disaccharides as lyoprotectants for *P. putida* KT2440. To select lyoprotectants that conferred high survival to *P. putida* KT2440 cells after freeze-drying, we tested the disaccharides, monosaccharides, polyalcohols, amino acids, and complex mixtures shown in Table 2 at the indicated concentrations. In this series of assays *P. putida* KT2440 cultures were grown in LB medium until the mid-exponential phase, when the cell density was ca. 10^8 CFU/ml. Aliquots of 5 ml were harvested by centrifugation, and cells were suspended in 5 ml of the indicated lyoprotectant. Before and after lyophilization the number of

TABLE 2. Effect of different lyoprotectants on the survival of *P. putida* KT2440 upon lyophilization

Compound	Concn (μM [or %])	Characteristic	Mean BSR (SD)
Lactose	0.20	Disaccharide	80.35 (1.63)
Maltose	0.20	Disaccharide	83.73 (1.18)
Sucrose	0.20	Disaccharide	78.03 (2.16)
Trehalose	0.13	Disaccharide	83.72 (1.51)
Fructose	0.20	Monosaccharide	65.32 (5.79)
Galactose	0.20	Monosaccharide	69.80 (3.83)
Glucose	0.20	Monosaccharide	67.80 (2.11)
Manose	0.20	Monosaccharide	71.68 (1.70)
Manitol	0.20	Polyalcohol	42.34 (6.76)
Myoinositol	0.27 (5%)	Polyalcohol	27.92 (4.77)
Sorbitol	0.27	Polyalcohol	61.69 (1.31)
Citrate	0.25	Tricarboxylic acid	41.82 (5.39)
Betaine	0.2	Amino acid	38.33 (6.20)
Glycine	0.2	Amino acid	23.23 (4.89)
Lysine	0.2	Amino acid	30.92 (1.08)
Proline	0.2	Amino acid	40.04 (4.77)
LB medium		Complex mixture	56.04 (5.81)
Skimmed milk	(20%)	Complex mixture	67.01 (1.83)

[a] The BSR is the ratio (log CFU/ml after freeze-dry/log CFU/ml before freeze-dry) × 100. Each value represents the average of five independent determinations.

TABLE 3. BSR of *P. putida* KT2440 at different growth phases[a]

Lyoprotectant	Mean BSR (SD) at:		
	Exponential (early) phase	Exponential (late) phase	Late stationary phase
Myoinositol	12.21 (8.00)	22.55 (7.5)	47.18 (2.00)
Trehalose	62.44 (5.0)	78.46 (5.0)	90.15 (1.50)

[a] *P. putida* KT2440 was grown on LB overnight. The culture was then diluted 100-fold. When it reached a turbidity at 660 nm of about 0.6 (early exponential phase), 1.2 (late exponential phase), or 4 (late stationary phase), aliquots were lyophilized with myoinositol or trehalose as the lyoprotectant.

viable cells was counted to estimate the BSR, as described in Materials and Methods. In general, we found that disaccharides were better lyoprotectants (BSR values of ca. 80) than monosaccharides (BSR values of ca. 68) and that these latter were better than polyalcohols, organic acids, LB, and amino acids. It should be noted that skimmed milk yielded a survival rate similar to that obtained with monosaccharides.

Effect of culture conditions and growth phase on the survival of *P. putida* KT2440 upon lyophilization. For the following series of assays we used two different lyoprotectants: trehalose, which gave a high BSR under our experimental conditions, and myoinositol, a lyoprotectant often used to freeze-dry microorganisms (13, 29). First, cells were grown in rich LB medium overnight and then diluted 100-fold in fresh LB medium, and the number of viable cells was determined at the early and late exponential phases and at the stationary phase. At these times aliquots of cultures were withdrawn and freeze-dried, and the BSR was determined. We found that regardless of the growth phase, the BSR was higher with trehalose than with myoinositol and that BSR was higher in cells that had reached the stationary phase than in cells in the exponential phase (Table 3).

We carried out assays similar to those described above but with cells grown in M9 minimal medium with different C sources, namely, betaine, benzoate, citrate, glucose, proline, and sucrose. We also found that, in general, survival was greater in cells that had reached the stationary phase than in cells in the exponential phase (not shown).

In our laboratory it has been a frequent practice to use solid LB or solid M9 minimal medium with citrate to grow cells for freeze-drying. We used 24-h-old plates that had been kept at 30°C and suspended cells for freeze-drying with trehalose or myoinositol. With trehalose the mean BSR value was 91 ± 3, and with myoinositol the mean value was 48 ± 1.

Regardless of how bacteria had been grown (liquid or solid medium) and whether cells were grown on rich or minimal medium, freeze-dried cells were stored under vacuum in the dark at 18 to 22°C. We found that survival decreased slightly with time and after 1 year the BSR of stored cells declined from 90 ± 2 to 80 ± 3.

Why do cells in the stationary phase survive better than cells in the exponential growth phase? The results described above suggested that culture conditions and the physiological state of *P. putida* cells notably influenced tolerance to the freeze-drying process, a finding that may be related to changes in the properties of the membranes with growth. We tested membrane fluidity of *P. putida* by determining the polarization value (P) in cells that had been grown on citrate as a carbon source or LB and that had reached the exponential or the stationary phase. Polarization values were significantly higher in cells in the stationary phase (0.313 ± 0.007) than in the exponential phase (0.288 ± 0.010), indicating that the membranes of *P. putida* were more rigid in the stationary phase than in the exponential growth phase. Membrane fluidity can be influenced by the composition of phospholipids. We reported before that the fatty acid composition of *P. putida* varied according to the growth phase. Ramos et al. (35) found that cells grown in LB medium to the exponential phase exhibited low levels of $C_{17:cyclopropane}$ (the most abundant cyclopropane fatty acid [CFA] found in *P. putida*), whereas CFA levels were high in the stationary phase. To test this potential correlation, we determined the fatty acid profile in cells growing on LB and M9 minimal media with citrate. In agreement with previous observations, $C_{17:cyclopropane}$ levels were higher in cells that had reached the stationary phase in M9 with citrate or LB. Concomitantly with the increase in $C_{17:cyclopropane}$, we noted an increase in BSR (Table 4).

Survival of mutants deficient in the synthesis of $C_{17:cyclopropane}$ in *P. putida* KT2440 upon freeze-drying. To gain further insight into the potential role of CFA in freeze-drying we generated mutants in the synthesis of $C_{17:cyclopropane}$. In gram-negative bacteria $C_{17:cyclopropane}$ is made from $C_{16:1cis}$ in a reaction catalyzed by CFA synthases (9). The genome of KT2440 had been sequenced previously and two potential CFA synthase genes were annotated (27). These genes are called *cfaA* and *cfaB* here, and their translated products corresponded to PP2734 and PP5365, respectively. We inactivated each of the genes by using *cfaA*:Km and *cfaB*:Gm mutant alleles and used them to generate each of the single mutants by allelic exchange.

We analyzed $C_{17:cyclopropane}$ levels in the mutant strains growing on citrate when cells had reached the exponential and the stationary phase. In the mutant strains $C_{17:cyclopropane}$ in the exponential phase was negligible, whereas in the stationary phase the level of $C_{17:cyclopropane}$ in the *cfaA* mutant was 20% of the total fatty acids versus ca. 30% in the wild type. Surprisingly, in the *cfaB* mutant $C_{17:cyclopropane}$ level in the stationary phase was less than 0.2% in LB, and it was not detected in cells grown on citrate as the sole carbon source (Table 5). We measured polarization values with the *P. putida* CFA-deficient strain and found that *P* values in the exponential and stationary

TABLE 4. Fatty acid profiles from *P. putida* KT2440 cells grown in different media and at different growth stages

Lipid	Mean % ± SD[a]			
	Exponential phase		Stationary phase	
	Citrate	LB	Citrate	LB
$C_{14:0}$	2.65 ± 0.28	1.77 ± 0.20	1.38 ± 0.11	2.09 ± 0.43
$C_{15:0}$	ND	0.06 ± 0.05	ND	0.81 ± 0.02
$C_{16:1,9cis}$	10.48 ± 1.31	23.74 ± 1.93	0.42 ± 0.19	3.09 ± 0.53
$C_{16:1,9trans}$	4.66 ± 0.30	6.47 ± 0.56	1.81 ± 0.8	1.24 ± 0.43
$C_{16:0}$	31.53 ± 0.68	35.45 ± 2.79	42.61 ± 0.91	33.22 ± 0.27
$C_{17:cyclopropane}$	0.71 ± 0.16	4.96 ± 0.64	30.05 ± 0.46	33.21 ± 1.19
$C_{17:0}$	0.44 ± 0.04	0.31 ± 0.09	0.70 ± 0.49	0.75 ± 0.03
$C_{18:1,11cis}$	11.75 ± 1.77	14.55 ± 0.83	6.30 ± 0.58	14.11 ± 0.74
$C_{18:1,11trans}$	0.91 ± 0.15	0.81 ± 0.06	ND	1.04 ± 0.26
$C_{18:0}$	36.84 ± 3.28	11.88 ± 6.13	10.24 ± 1.26	6.89 ± 0.40
$C_{19:cyclopropane}$	ND	ND	6.50 ± 0.87	4.15 ± 0.82
BSR	66.67 ± 2.88	79.23 ± 0.98	92.07 ± 2.20	91.28 ± 1.50

[a] Each value represents the average of three repetitions from independent experiments. BSR values are shown at the bottom of the table. ND, not detected.

TABLE 5. BSR and levels of $C_{17:cyclopropane}$ in *P. putida* KT2440 and several isogenic mutant strains

P. putida strain	Mean % (SD)[a]			
	Level of $C_{17:cyclopropane}$ at:		BSR at:	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase
KT2440	0.5 (0.2)	30.7 (1.5)	68.5 (2.21)	91.83 (1.63)
cfaA mutant	0.1 (0.1)	20.2 (3.60)	64.94 (6.3)	85.31 (1.23)
cfaB mutant	ND	ND	58.94 (5.3)	70.55 (1.60)
ropS (miniTn*5*::*lux*) mutant	0.1 (0.1)	7.0 (1.0)	66.48 (2.7)	73.39 (1.99)

[a] Each value represents the average of three repetitions from independent experiments. Cells were grown in M9 minimal medium with 20 mM citrate. The turbidity of the cultures in the exponential phase was around 0.3 ± 0.05 at 660 nm, whereas in the stationary phase the turbidity at 660 nm was 2.5 ± 0.2. ND, not detected.

phase were similar to those observed for the wild type. In this mutant strain the increase in rigidity observed could be due to a fivefold increase in the levels of $C_{16:1trans}$ (not shown). We calculated the BSR of each strain with a mutation in the *cfa genes*, in cells in the exponential and stationary phase. For the *cfaB* mutant, regardless of the growth phase, BSR values were much lower than those of the parental strain. For the *cfaA* mutant in the exponential phase, the BSR values were similar to those of the wild-type strain; however, when cells reached the stationary phase the values were slightly lower (Table 5). It then follows that BSR values correlate well with $C_{17:cyclopropane}$ levels.

In *E. coli cfa* transcription is mediated by the *rpoS* sigma factor when cells reach the stationary phase (11, 45). This also seems to be the case in KT2440 (P. Bernal et al., unpublished). We tested CFA levels in an *rpoS* KT2440 mutant background and found that the *rpoS* mutant produced very small amounts of $C_{17:cyclopropane}$ in the stationary phase and that its BSR values were concomitantly lower than those of the wild-type strain.

DISCUSSION

Freeze-drying is the method often used for the preservation and long-term storage of bacterial cells (29); however, it has negative effects on the viability of many cell types (24). It is known that lyophilization of bacteria results in the loss of viability during the process (32) and that such loss is influenced by the lyoprotectant used (12, 22, 44), the initial cell density (8, 28), and the physiological state of the cells (6, 13). Several compounds and mixtures were used as lyoprotectants for *P. putida* KT2440 in the present study. We found that viability was affected to a certain extent under all conditions compared here but that disaccharides, e.g., trehalose, maltose, and lactose, were the best lyoprotectants. This finding is in agreement with observations reported in experiments with other microorganisms (*Escherichia coli*, *Bacillus thuringiensis*, and *Pseudomonas chlororaphis*) that were protected during lyophilization by disaccharides (22, 23, 28). The molecular mechanism behind the protective effect of disaccharides during freeze-drying is unknown, although it has been suggested that these chemicals protect membrane integrity by replacing water molecules in the membrane during the freeze-drying process (23).

With disaccharides as lyoprotectants for *P. putida* KT2440, we observed that survival was notably influenced by the physiological state of bacteria. In particular, cells in the stationary phase survived better than those in the exponential phase. In the stationary phase bacteria accumulate compatible solutes (21, 31, 40) and produced stress proteins under carbon starvation conditions (17, 28). We cannot rule out these possibilities of protecting the cells against damage during lyophilization; nevertheless, we suggest that membrane alterations are the main factor responsible for the increase in BSR. We found that the increase in BSR in the stationary phase correlated with an increase in $C_{17:cyclopropane}$ levels. The presence of high levels of $C_{17:cyclopropane}$ in the stationary phase are in agreement with a recent report by Härtig et al. (16), although we have noticed higher levels of $C_{18:0}$ in the same strain under the growth conditions we used. Cyclopropane fatty acids seem to stabilize membrane lipids against turnover and degradation (23). Several reports have indicated that CFAs are involved in stress tolerance.

Zhao et al. (47) reported that overexpression of a cloned cyclopropane fatty acid synthase in *Clostridium* sp. influenced butanol resistance and in *E. coli* and *Streptococcus* sp. CFAs were said to be involved in acid stress since *E. coli cfa* mutants were shown to be more sensitive to stress (7, 33). In *E. coli* CFA deficiency also led to increased sensitivity to recurring freeze-thaw cycles (42). *Azotobacter vinelandii* synthesizes $C_{17:cyclopropane}$ fatty acid during differentiation into cysts as a mechanism of resistance to water and other environmental stresses (41). It seems then that under certain conditions CFA can function as a resistance barrier against environmental stresses. We reasoned that if $C_{17:cyclopropane}$ fatty acid was involved in increased tolerance to lyophilization in *P. putida*, mutants deficient in the synthesis of $C_{17:cyclopropane}$ fatty acids should be less tolerant to freeze-drying than wild-type cells. First, we showed that single mutants in *cfaA* or *cfaB* grew as fast as the wild type in LB rich medium. We then tested the mutants' response to lyophilization. Our finding that the single *cfaB* mutant, which produced very low levels of $C_{17:cyclopropane}$, was hypersensitive to lyophilization even when it reached the stationary phase of growth on LB medium, supports a role of this fatty acid in tolerance to lyophilization. The *cfaA* single mutant that synthesized ca. 66% of the total $C_{17:cyclopropane}$ fatty acid produced by the wild type was more sensitive than the parental strain to lyophilization but much more resistant than the *cfaB* mutant. The increased resistance to freeze-drying offered by $C_{17:cyclopropane}$ fatty acid can be interpreted as evidence for its role in the alteration of the membrane's chemical properties. This is in consonance with the increased resistance of CFA to ozonolysis, singlet oxygen, and mild oxidative treatments (14, 15).

In *P. putida* CFA synthesis occurred in the stationary phase, and expression of *cfa* genes seems to be mediated by the RpoS sigma factor. This was suggested by the finding that an *rpoS* knockout mutant produced very low levels of CFA. In consonance with the low levels of $C_{17:cyclopropane}$ fatty acids, the RpoS mutant was also hypersensitive to lyophilization. In *E. coli* the expression of the *cfa* genes is influenced not only by RpoS but also by the Crp and Fur proteins (46). Under our

growth conditions a role for iron in CFA synthesis can be ruled out, since KT2440 was grown in iron-rich medium, and no iron siderophores were detected in the medium. Catabolite repression might play a role in *cfa* gene expression, but in *P. putida* the intimate mechanisms of catabolite repression have not been elucidated (1, 10). In the near future we plan to perform detailed transcriptional studies of *cfa* synthesis in different genetic backgrounds.

In short, our results indicate that cyclopropane fatty acids are important in *P. putida* KT2440 for survival in freeze-drying processes and that the highest survival rates in this strain are achieved with disaccharides as lyoprotectants.

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