Journal of General Microbiology (1988), **134**, 433–440. *Printed in Great Britain*

Chromosomal Mapping and Cloning of the Lipase Gene of *Pseudomonas aeruginosa*

By SUSANNE WOHLFARTH* AND ULRICH K. WINKLER

Ruhr-Universität Bochum, Lehrstuhl für Biologie der Mikroorganismen, D-4630 Bochum, FRG

(*Received 13 April 1987; revised 24 September 1987*)

Various mutants (*lip*) of *Pseudomonas aeruginosa* PAO 2302 that lacked extracellular lipase activity were isolated. They were selected on a calcium–triolein agar. The phenotypic characteristics of two of these mutants suggested that they were defective in the gene coding for lipase: both *lip* mutants produced no lipase in liquid- and on solid medium. They were non-pleiotropic with regard to various other exoproducts. None of the mutants released any putatively cell-bound lipase after treatment of cells with Triton X-100 or alginate. The electrophoretic protein- and LPS-profiles of outer membranes derived from *lip* mutants and the parental strain were identical. The *lip* locus was mapped on the chromosome of *P. aeruginosa* PAO 1 by FP5- and R68.45-mediated crossings and by transduction with phage G101. The *lip* locus was cotransduced with *pyrF* only (60%) indicating a map position at about 57 min. The lipase gene was cloned on a 3·1 kb *Sal*I fragment using vector pKT248. The newly constructed plasmid was able to complement the lipase deficiency of the two *lip* mutants of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen causing life-threatening infections especially in patients suffering from cystic fibrosis or having a compromised immune system. Several extracellular proteins produced by *P. aeruginosa*, e.g. exotoxin A, elastase, phospholipase C and exoenzyme S, were considered as virulence factors (Govan & Harris, 1986; Winkler *et al.*, 1985). The pathogenic role of the extracellular lipase is unknown so far. Besides this clinical aspect, the lipase of *P. aeruginosa* is also of interest in biotechnology as it specifically catalyses trans- and interesterifications (Lazar, 1985).

The lipase of *P. aeruginosa* PAC 1R is a lipid-hydrolysing enzyme (EC 3.1.1.3) excreted into the medium during the late exponential growth phase. It has an apparent M_r of 29000 and the native enzyme is strongly associated with lipopolysaccharide (LPS) (Stuer *et al.*, 1986).

In this paper we report on the physiological characterization of newly isolated lipase-deficient (*lip*) mutants from *P. aeruginosa* PAO 2302 and on the chromosomal mapping and cloning of the corresponding gene. Some of the results described were presented at the EMBO workshop '*Genetic Manipulation of Pseudomonads – Applications in Biotechnology and Medicine*', Geneva, 31 August to 4 September, 1986.

METHODS

Bacteria, plasmids and phage. All *Pseudomonas aeruginosa* strains used were derivatives of wild-type *P. aeruginosa* PAO 1 (ATCC 15692). They are listed in Table 1.

Auxotrophic donors carrying the conjugative resistance plasmids FP5 (Matsumoto & Tazaki, 1973) or R68.45 (Haas & Holloway, 1976) were constructed as described by Haas & Holloway (1976) and Haas *et al.* (1977) using *P. aeruginosa* PAO 4020 (FP5) or *P. aeruginosa* PAO 25 (R68.45) as donors of the desired plasmid. For transductional analysis phage G101 was used (Holloway & van de Putte, 1968). In cloning experiments *Escherichia*

Abbreviations: NB, nutrient broth; LB, Luria broth; CT, calcium–triolein; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Table 1. *Strains of P. aeruginosa used in this study*

Strain	Genotype*	Reference	
PAO 1	Prototroph	Holloway *et al.* (1979)	
PAO 8	*met-28 ilv-202 str-1*	Isaac & Holloway (1968)	
PAO 25	*argF-10 leu-10*	Haas & Holloway (1976)	
PAO 166	*leu-10 pyrF*	Royle *et al.* (1981)	
PAO 325	*argB-18 lys-57*	Isaac & Holloway (1972)	
PAO 388	*aro-1 pyrF69 pur-70*	Royle *et al.* (1981)	
PAO 436	*ser-3 bla-436*	Krishnapillai *et al.* (1981)	
PAO 2302	*catA1 met-9020 puuA1*	Matsumoto *et al.* (1978)	
PAO 4020	*nir-9006 his-9004 str-9002*	Matsumoto *et al.* (1981)	
PAO 4044	*met-9020 catA1 nar-9011 mtu-9002 tyu-9030 dcu-9041*	Matsumoto *et al.* (1981)	
29-1	*met-9020 catA1 puuA1 lip1*	NG derivative of PAO 2302	This study
29-4	*met-9020 catA1 puuA1 lip1 arg*	NG derivative of 29-1	This study
6-1	*met-9020 catA1 puuA1 lip2*	NG derivative of PAO 2302	This study
6-4	*met-9020 catA1 puuA1 lip2 arg*	NG derivative of 6-1	This study
Plasmid	Phenotype		
R68.45	$Cb^R Km^R Tc^R$	Haas & Holloway (1976)	
FP5	Hg^R	Matsumoto *et al.* (1981)	
pKT248	$Sm^R Cm^R$	Bagdasarian *et al.* (1981)	

* Symbols were used according to Holloway *et al.* (1979); *lip*, deficiency in extracellular lipase activity.

coli–P. aeruginosa shuttle-vector pKT248 ($Sm^R Cm^R$) was used (Bagdasarian *et al.*, 1981). *E. coli* strains SK 1108 (F^- *pyrF* Km^R *gal thi sup* Ti-R *hsdR-4 endA sbcB-15*) from Donovan & Kushner (1983) and JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac–proAB*) F′ *traD36 proAB lacI*^{q}ZΔM15) (Yanisch-Perron *et al.*, 1985) were used as heterologous cloning hosts.

Media and growth conditions. Strains of *P. aeruginosa* were usually grown in nutrient broth (NB; Difco) under aeration at 37 °C. Three different minimal selection media (each solidified with 1·5%, w/v, Bacto-agar) were used. (i) Minimal medium M9 (Maniatis *et al.*, 1982) when selecting for prototrophic revertants of amino acid auxotrophs. When necessary, amino acids were added to the medium at a final concentration of 0·1 mM. (ii) Minimal medium according to Ornston & Stanier (1966) when selecting for cells utilizing unusual carbon sources such as mannitol, benzoic acid, tyrosine and diaminopimelic acid. These carbon sources were added to a final concentration of 10 mM. (iii) Minimal medium according to Davis & Mingioli (1950) to select for bacteria independent of adenine, uracil and aromatic amino acids; final concentrations of supplements were 0·1 mM.

To distinguish between clones of lipase-excreting (*lip*$^+$) and -defective (*lip*) bacteria, a newly developed plate test using calcium–triolein (CT) agar was applied. Demineralized water (45 ml) and purified olive oil (5 ml) were vigorously stirred for 15 min on a magnetic stirrer. This mixture and 5 ml of a 1% (w/v) $CaCl_2$ solution were added to 450 ml NB agar (containing 4 g NB, 2 g NaCl and 7·5 g agar). During continuous stirring for another 5–10 min the solution was aseptically adjusted to pH 7·5. All solutions were previously sterilized by autoclaving (20 min; 200 kPa). The agar plates contained little oil droplets on the surface of the solidified medium. Inoculated plates were incubated at 30 °C.

For phenotypic characterization of *lip* mutants different agar plate tests were used. (i) Hydrolysis of triolein. For medium composition see above. *lip*$^+$ colonies showed white crystals (calcium oleate) on top whereas *lip* colonies did not. (ii) Hydrolysis of Tween 80. Test conditions according to Howe & Ward (1976). (iii) Hydrolysis of gelatin. NB agar contained 0·4% Bacto-gelatin. Hydrolysis could be detected as clear zones surrounding the colonies after pouring 0·5 M-sulphuric acid (saturated with Na_2SO_4) onto the agar. (iv) Hydrolysis of elastin. NB agar contained 0·3% elastin (Sigma). Hydrolysis resulted in clear zones around the colonies. (v) Hydrolysis of lecithin. One egg-yolk was aseptically mixed into 250 ml NB agar. Hydrolysis was indicated by the formation of white precipitates under the colonies (Gerhardt *et al.*, 1981). (vi) Pyocyanin production. This was best on peptone–glycerol agar (Gerhardt *et al.*, 1981). Fluorescein production was assayed on NB agar, pH 6·0, supplemented with $MgSO_4$ (0·15%). The latter showed yellow fluorescence when irradiated with UV light (about 265 nm) (Gerhardt *et al.*, 1981).

Additionally, we used Luria broth (LB) (Maniatis *et al.*, 1982) and LB agar (solidified with 1·5%, w/v, agar) while working with *E. coli* strains. Streptomycin sulphate (50 μg ml^{-1}) or chloramphenicol (20 μg ml^{-1}) were added to LB, NB and CT agar for selection purposes.

Mutagenesis. P. aeruginosa PAO 2302 was grown in NB to late exponential growth phase, washed in 20 mM-

potassium phosphate buffer, pH 6·0, and treated with 50 μg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) ml^{-1} for 7 min at 37 °C. Frequency of survivors was about 15%.

Conjugation and transduction. Conjugations using mobilization plasmid FP5 were done in NB according to Matsumoto *et al.* (1981). Both donor and recipient strain were grown to mid-exponential growth phase and usually mixed in volume ratios of 1 : 1; 1 : 2 or 1 : 5. Matings were done without any agitation at 37 °C for 3 h. Samples (0·1 ml) of washed (0·9% NaCl) cells were plated onto selective media.

Using plasmid R68.45, matings were done on plates. Donor and recipient cells of exponential growth phase were concentrated to 6×10^9 cells ml^{-1} in 0·9% NaCl before they were mixed and plated onto selective media (Stanisich & Holloway, 1972).

Transduction with phage G101 was done according to Matsumoto *et al.* (1981). Cells from exponential growth phase cultures were concentrated to 10^9 cells ml^{-1} in TNM buffer (10 mM-Tris/HCl, pH 7·4, 150 mM-NaCl, 10 mM-$MgSO_4$) and phage was added to give a multiplicity of infection of five to seven. After preadsorption the cells were concentrated tenfold and plated onto selective media.

Preparation of outer membranes. This was done using the Sarkosyl method of Lambert & Booth (1982). The membrane proteins were solubilized at 90 °C for 10 min in an SDS-buffer system. They were electrophoretically separated on a 14% (w/v) acrylamide/0·12% bisacrylamide gel and then stained with Coomassie brilliant blue G. Electrophoretic LPS-profiles were obtained by SDS solubilization (see above) and proteinase K digestion (0·4 mg ml^{-1}, 60 min at 50 °C) of outer membrane preparations examined by electrophoresis on 12% (w/v) acrylamide/5% (w/v) bisacrylamide gels and silver staining (Tsai & Frasch, 1982).

Enzyme assays. Lipase activity was assayed spectrophotometrically using *p*-nitrophenyl palmitate as substrate (Winkler & Stuckmann, 1979). In addition, a bioluminescent lipase assay (Ulitzur, 1979) was used to measure very low lipase activities. In this test the enzymic release of myristic acid from trimyristin was followed by the fatty-acid-dependent bioluminescence of a *dim* mutant of *Vibrio harveyi*. All lipase assays were done at least twice. Esterase activity was assayed spectrophotometrically with palmitoyl-CoA as substrate (Ohkawa *et al.*, 1979).

Molecular genetic methods. Chromosomal DNA of *P. aeruginosa* PAO 1 was prepared as described by Maniatis *et al.* (1982). Plasmids were isolated according to Birnboim & Doly (1979) and further purified by centrifugation to equilibrium in ethidium-bromide–CsCl gradients. Restriction endonuclease digestions and ligations were done as recommended by the supplier (BRL). *E. coli* was transformed by a $CaCl_2$-method described by Maniatis *et al.* (1982). For the transformation of *P. aeruginosa* we used cells from early-exponential growth phase cultures washed twice with 150 mM-$MgCl_2$. After overnight incubation in 150 mM-$MgCl_2$ at 4 °C cells were competent (Olsen *et al.*, 1982).

RESULTS

Isolation and phenotypic characterization of lip mutants

P. aeruginosa PAO 2302 was treated with NG in two independent mutagenesis batches. About 0·4% of the total number of colonies screened ($n \approx 16000$) did not show lipase activity on CT agar. Many of these were pleiotropic mutants as were those described by Wretlind & Pavlovskis (1984) and were therefore not investigated.

Two independent *lip* mutants, strains 29-1 and 6-1 seemed to be non-pleiotropic and were phenotypically characterized in detail. When tested on CT agar neither produced an extracellular lipase. Enzyme assays confirmed that both mutants also did not produce extracellular and cell-bound lipase in liquid media – neither in minimal medium M9 nor in NB at 30 °C or 37 °C. They still had a cell-bound esterase able to hydrolyse Tween 80, *p*-nitrophenyl palmitate and palmitoyl-CoA (Table 2).

When *P. aeruginosa* PAO 2302 was incubated in the presence of 0·2% alginate the extracellular lipase activity increased by a factor of about eight (Table 3), as expected from experiments with other strains [alginate is assumed to detach cell-bound lipase (Wingender & Winkler, 1984)]. However, neither of the *lip* mutants showed any response to alginate (Table 3). Likewise, treatment of *lip* mutants with Triton X-100 (0·02%), which solubilizes some membrane-bound proteins, did not release any lipase activity from the cells.

lip mutants 29-1 and 6-1 were of the non-pleiotropic type with respect to their ability to hydrolyse gelatin, elastin and lecithin (Table 4). Formation of the pigments pyocyanin and fluorescein was not influenced either (Table 4). These results suggested that no central excretion pathway of extracellular products was mutationally altered.

The two *lip* mutants and the corresponding parental strain PAO 2302 showed the same electrophoretic banding pattern when their outer membrane proteins and LPSs were studied by

Table 2. *Lipase and esterase activities of P. aeruginosa PAO 2302 and the two lip mutants*

Cells grown in NB medium at 30 °C for 16 h to stationary phase were removed from the culture medium by centrifugation. Enzyme activities were measured in the cleared supernatants. Substrates were trimyristine (*a*), *p*-nitrophenyl palmitate (*b*) and palmitoyl-CoA (*c*). All enzyme activities were standardized for equal cell density (OD_{580} = 1).

	Enzyme activity (nmol min^{-1} ml^{-1})				
	Extracellular			Cell-bound	
Strain	(*a*) Lipase	(*b*) Lipase and Esterase	(*c*) Esterase	(*b*) Lipase and Esterase	(*c*) Esterase
Parental strain PAO 2302	24	145	0·950	47·6	7
lip mutants					
29-1	0·001	2·6	0·770	8·7	4·7
6-1	0·042	3·2	0·785	9·1	4·4

Table 3. *Extracellular lipase activity of P. aeruginosa PAO 2302 and the two lip mutants in the absence and presence of exogenous alginate*

Lipase activity was measured in the supernatants of bacterial cultures grown at 30 °C to stationary phase (23 h) in M9 minimal medium supplemented with 0·1 mM-methionine. Enzyme activities (*p*-nitrophenyl palmitate as substrate) were standardized to equal cell density (OD_{580} = 1) and corrected for quenching due to bacterial pigments.

	Lipase activity (nmol min^{-1} ml^{-1})		
Strain	− Alginate	+ Alginate*	Stimulation factor
Parental strain PAO 2302	59	447	7·6
lip mutants			
29-1	1·7	1·2	0
6-1	2·2	1·0	0

* Alginate (Manucol LB) was added to a final concentration of 0·2%.

Table 4. *Phenotypic characterization of P. aeruginosa PAO 2302 and the two lip derivatives based on agar plate tests*

+, Wild-type activity; −, no activity; +/−, very little activity.

		lip mutants	
	Parental strain PAO 2302	29-1	6-1
Hydrolysis of:			
triolein	+	−	−
Tween 80	+	+/−	+/−
gelatin	+	+	+
elastin	+	+	+
lecithin	+	+	+
Formation of:			
pyocyanin	+	+	+
fluorescein	+	+	+

SDS-PAGE (Fig. 1). This indicated that the missing extracellular lipase activity of the mutants was not due to structural changes of outer membrane components involved in exoenzyme excretion.

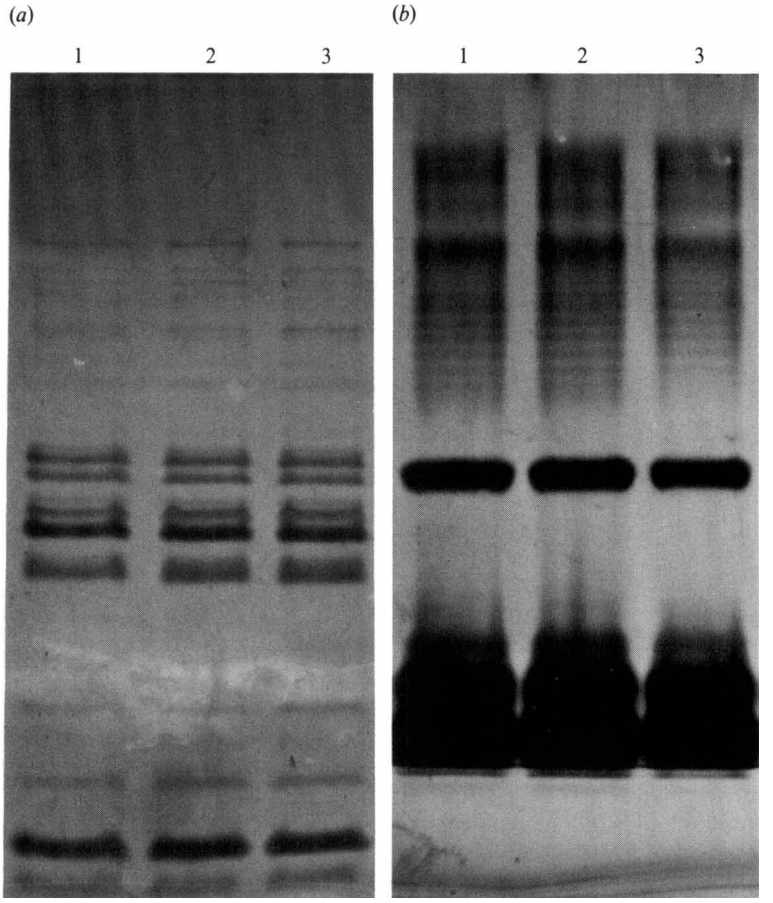

Fig. 1. PAGE of outer membrane proteins (*a*) and LPS profile (*b*) of *P. aeruginosa* PAO 2302 (lane 1) and the *lip* derivatives 29-1 (lane 2) and 6-1 (lane 3).

Genetic mapping of the lip locus

The chromosomal location of the mutations in both *lip* mutants was determined by crossing and transduction experiments.

Crossing experiments using plasmid FP5 for chromosome mobilization (Table 5*a*) allowed a rough localization of *lip* loci of both mutants at later than 50 min on the chromosome of *P. aeruginosa* PAO 1 (we refer to the map of *P. aeruginosa* PAO 1 chromosome published by Holloway & Matsumoto in '*Genetic Maps 1984*'). A more detailed map position was obtained by R68.45-mediated crossings (Table 5*b*). The loci of both *lip* mutants were found to be in the 55–64 min region on the bacterial chromosome with the closest linkage (93–95%) to the selective marker *pyrF*.

Bacteriophage G101 was used for transductional analysis. Selecting for seven different markers [*leu-10* (54 min); *met-9011* (55 min); *pyrF63* (57 min); *dcu-9041* (60 min); *catA1* (64 min); *mtu-9002* (70 min); *tyu-9030* (75 min)] gave cotransduction frequencies of 58% (29-1) and 60% (6-1) of the non-selective *lip* marker with *pyrF63* only.

Cloning of the lipase gene

P. aeruginosa PAO 1 DNA was partially digested with restriction endonuclease *Sal*I and ligated into the single *Sal*I site of the shuttle-vector pKT248. Transformants of *E. coli* SK 1108 were selected for streptomycin resistance and contraselected for chloramphenicol sensitivity.

Table 5. *Linkage of the lip locus of mutants 29-1 and 6-1 to selective markers of P. aeruginosa PAO in FP5- and R68.45-mediated crossings*

Selective marker	Map location (min)	*lip* linkage (%) 29-1	6-1
(*a*) FP5-mediated crossings			
argF	45	0	0
leu-10	54	3	0
met-9011	55	59	57
catA1/met-9011	55/64	95	95
(*b*) R68.45-mediated crossings			
argB	21	NT	0
lys-57	20	NT	0
ser-3	30	NT	0
aro-1	53	2	10
leu-10	54	1	2
met-9011	55	24	25
pyrF63	57	93	95
aro-1/pyrF63	53/57	92	90
dcu-9041	60	16	27
catA1	64	18	14
catA1/met-9011	55/64	96	96
mtu-9002	70	NT	4
tyu-9030	75	0	1
pur-70	89	0	0

NT, Not tested.

Six-thousand clones were divided into 120 portions each consisting of 50 clones. Mixed plasmid preparations were obtained from each portion and used to transform *P. aeruginosa lip* mutant 6-1. Selection was made on CT agar supplemented with streptomycin.

One recombinant plasmid named pSW1 could be selected with a *Sal*I insert of 3·1 kb composed of a 1·3 kb, a 0·97 kb and a 0·76 kb *Sal*I subfragment.

This plasmid fully complemented the *lip* phenotype of both lipase-defective mutants (29-1 and 6-1) of *P. aeruginosa*. With regard to lipase excretion no differences could be detected between the parental strain PAO 2302 and the *lip* mutant derivatives carrying plasmid pSW1. In *E. coli* JM109(pSW1), however, no lipase activity was found extracellularly at different growth phases. When cells of *E. coli* JM109(pSW1) were disrupted by sonication the intracellular lipase activity was below 1 nmol min^{-1} ml^{-1} using *p*-nitrophenyl palmitate as enzyme substrate. Experiments to subclone the fragment and insert it into a strong *E. coli* expression vector are under way.

Nevertheless, using other selection media we found two independent recombinant plasmids with a 5·3 kb or a 10 kb *Sal*I insert complementing the *pyrF* phenotype of *E. coli* SK1108.

DISCUSSION

Phenotypic differences were used by Wretlind & Pavlovskis (1984) to distinguish between two classes of exoproduct-deficient mutants of *P. aeruginosa*: class I are mutants defective in exporting exoproducts, and class II are mutants defective in the regulation of exoproduct formation. The *lip* mutants 29-1 and 6-1 we have isolated and phenotypically characterized do not belong to either of these classes. Both mutants were not pleiotropic with regard to the formation of various exoproducts. Neither showed any extracellular lipase activity, on solid media or in broth cultures. Compartmental analysis of our mutants did not indicate any intracellular accumulation of lipase activity (data not shown). Furthermore, treatment of the mutants either with Triton X-100 or with alginate did not cause any release of putatively cell-bound lipase activity into the medium. The electrophoretic banding pattern of proteins and LPSs were alike when the outer membranes from *lip* mutants and their parental strain were compared. In summary, the data suggested that our *lip* mutants did arise by mutations in the structural gene for lipase.

The results of conjugational and transductional analysis of *lip* mutants 29-1 and 6-1 were in good agreement with the above conclusion because the new mutant loci were not identical with any loci so far published and known to be involved in regulation and/or secretion of exoproducts in *P. aeruginosa*. The mutations of both our *lip* mutants were located at map position 57 min on the chromosome, closely linked and cotransduced with *pyrF63* only. Mutations interfering with regulation and/or secretion of exoproducts, however, are located at 0 min (*xcp-1*), 35 min (*xcp-2*, *xcp-3*), 40 min (*xcp-4*), 55 min (*xcp-5*, *xch-1*, *xch-2*) and 67 min (*xcp-6*) (Wretlind & Pavlovskis, 1984; Björklind *et al.*, 1985; Filloux *et al.*, 1987).

Structural genes for exoproducts of *P. aeruginosa* neither prefer a certain map position on the chromosome nor do they tend to cluster as indicated by the following data: at 20 min – *plcA*, *B* (phospholipase C) (Gray & Vasil, 1981); at 23 min – exoenzyme S (Nikas, 1986); at 57 min – *lip* (lipase) (this study); at 35 and 67 min – *pvd* (pyoverdine) (Hohnadel *et al.*, 1986); at 75 min – *lasA* (elastase) (Howe *et al.*, 1983); and at 85 min – *toxA* (exotoxin A) (Hanne *et al.*, 1983).

Using vector pKT248 it was possible to clone a 3·1 kb fragment of DNA from *P. aeruginosa* PAO 1 which was able to fully complement the lipase deficiency of *lip* mutants 29-1 and 6-1. This insert probably contained the structural gene for lipase. After transfer of this new plasmid (called pSW1) to *E. coli* JM109 traces of lipase activity could be detected in the cells. The low expression of the lipase gene was not surprising because of the poor recognition of *Pseudomonas* promoters in *E. coli* (Jeenes *et al.*, 1986). Subcloning of the lipase gene and sequencing will enable us to see whether the base sequence will meet the expectations based on the apparent M_r (29000) of the lipase estimated from SDS-PAGE (Stuer *et al.*, 1986).

When preparing this manuscript a recent paper by Odera *et al.* (1986) came to our attention reporting the cloning of a lipase gene from a newly isolated soil bacterium identified as *P. aeruginosa*. These authors used plasmid RP4 : Mu*cts*62 for *in vivo* cloning and found good expression of the cloned lipase gene also in *E. coli*. Furthermore, Kugimiya *et al.* (1986) published work on the cloning of a lipase gene from *P. fragi*. Preliminary comparison of some data from Kugimiya *et al.* (1986) and from our group (Stuer *et al.*, 1986) suggests that *P. aeruginosa* and *P. fragi* produce different lipases.

We gratefully acknowledge the excellent technical assistance of Mrs Petra Wahl. We thank Drs D. Haas, B. W. Holloway, S. Kushner, H. Matsumoto, K. Timmis, S. Ulitzur and B. Wretlind for kindly supplying us with bacterial strains, plasmids and phages. Part of this work was financially supported by the Deutsche Gesellschaft zur Bekämpfung der Mucoviscidose.

REFERENCES

BAGDASARIAN, M., LURZ, R., RÜCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host–vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.

BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.

BJÖRKLIND, A., WRETLIND, B., MÖLLEGÅRD, I., SCHAD, P. A., IGLEWSKI, B. H. & COX, C. D. (1985). Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants that hyperproduce exoproteins. *Journal of Bacteriology* **162**, 1329–1331.

DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. *Journal of Bacteriology* **60**, 17–28.

DONOVAN, W. P. & KUSHNER, S. R. (1983). Cloning and physical analysis of the *pyrF* gene (coding for orotidine-5′-phosphate decarboxylase) from *Escherichia coli* K-12. *Gene* **25**, 39–48.

FILLOUX, A., MURGIER, M., WRETLIND, B. & LAZDUNSKI, A. (1987). Characterization of two *Pseudomonas aeruginosa* mutants with defective secretion of extracellular proteins and comparison with other mutants. *FEMS Microbiology Letters* **40**, 159–163.

GERHARDT, P., MURRAY, R. G. E., COSTILOW, R. N., NESTER, E. W., WOOD, W. A., KRIEG, N. R. & PHILLIPS, G. B. (1981). *Manual of Methods for General Bacteriology*. Washington, DC: American Society for Microbiology.

GOVAN, J. R. W. & HARRIS, G. S. (1986). *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiological Sciences* **3**, 302–308.

GRAY, G. L. & VASIL, M. L. (1981). Mapping of a gene controlling the production of phospholipase C and alkaline phosphatase in *Pseudomonas aeruginosa*. *Molecular and General Genetics* **183**, 403–405.

HAAS, D. & HOLLOWAY, B. W. (1976). R factor variants with enhanced sex factor activity in *Pseudomonas aeruginosa*. *Molecular and General Genetics* **144**, 243–251.

HAAS, D., HOLLOWAY, B. W., SCHAMBÖCK, A. & LEISINGER, T. (1977). The genetic organization of

arginine biosynthesis in *Pseudomonas aeruginosa*. *Molecular and General Genetics* **154**, 7–22.

HANNE, L. F., HOWE, T. R. & IGLEWSKI, B. H. (1983). Locus of the *Pseudomonas aeruginosa* toxin A gene. *Journal of Bacteriology* **154**, 383–386.

HOHNADEL, D., HAAS, D. & MEYER, J.-M. (1986). Mapping of mutations affecting pyoverdine production in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **36**, 195–199.

HOLLOWAY, B. W. & MATSUMOTO, H. (1984). *Pseudomonas aeruginosa*. In *Genetic Maps 1984*, vol. 3, pp. 194–197. Edited by S. J. O'Brien. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

HOLLOWAY, B. W. & VAN DE PUTTE, P. (1968). Lysogeny and bacterial recombination. In *Replication and Recombination of Genetic Material*, pp. 175–183. Edited by W. J. Peacock & R. D. Brock. Australian Academy of Science.

HOLLOWAY, B. W., KRISHNAPILLAI, V. & MORGAN, A. F. (1979). Chromosomal genetics of *Pseudomonas*. *Microbiological Reviews* **43**, 73–102.

HOWE, T. G. B. & WARD, J. M. (1976). The utilization of Tween 80 as carbon source by *Pseudomonas*. *Journal of General Microbiology* **92**, 234–235.

HOWE, T. R., WRETLIND, B. & IGLEWSKI, B. H. (1983). Comparison of two methods of genetic exchange in determination of the genetic locus of the structural gene for *Pseudomonas aeruginosa* elastase. *Journal of Bacteriology* **156**, 58–61.

ISAAC, J. H. & HOLLOWAY, B. W. (1968). Control of pyrimidine biosynthesis in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **96**, 1732–1741.

ISAAC, J. H. & HOLLOWAY, B. W. (1972). Control of arginine biosynthesis in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **73**, 427–438.

JEENES, D. J., SOLDATI, L., BAUR, H., WATSON, J. M., MERCENIER, A., REIMMANN, C., LEISINGER, T. & HAAS, D. (1986). Expression of biosynthetic genes from *Pseudomonas aeruginosa* and *Escherichia coli* in the heterologous host. *Molecular and General Genetics* **203**, 421–429.

KRISHNAPILLAI, V., ROYLE, P. & LEHRER, J. (1981). Insertions of the transposon Tn*1* into the *Pseudomonas aeruginosa* chromosome. *Genetics* **97**, 495–511.

KUGIMIYA, W., OTANI, Y., HASHIMOTO, Y. & TAKAGI, Y. (1986). Molecular cloning and nucleotide sequence of the lipase gene from *Pseudomonas fragi*. *Biochemical and Biophysical Research Communications* **141**, 185–190.

LAMBERT, P. A. & BOOTH, B. R. (1982). Exposure of outer membrane proteins on the surface of *Pseudomonas aeruginosa* PAO 1 revealed by labelling with [^{125}I]lactoperoxidase. *FEMS Microbiology Letters* **14**, 43–45.

LAZAR, G. (1985). Estersynthesen mit Lipasen. *Fette Seifen Anstrichmittel* **87**, 394–400.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

MATSUMOTO, H. & TAZAKI, T. (1973). FP5 factor, an undescribed sex factor of *Pseudomonas aeruginosa*. *Japanese Journal of Microbiology* **17**, 409–417.

MATSUMOTO, H., NAKAZAWA, T., OHTA, S. & TERAWAKI, Y. (1981). Chromosomal locations of *catA*, *pobA*, *pcaA*, *dcu* and *chu* genes in *Pseudomonas aeruginosa*. *Genetical Research* **38**, 251–266.

MATSUMOTO, H., OHTA, S., KOBAYASHI, R. & TERAWAKI, Y. (1978). Chromosomal location of genes participating in the degradation of purines in *Pseudomonas aeruginosa*. *Molecular and General Genetics* **167**, 165–176.

NIKAS, T. I. (1986). Genetic mapping of a gene regulating the production of exoenzyme S in *Pseudomonas aeruginosa* PAO. In *Abstracts of EMBO-workshop: Genetic Manipulation of Pseudomonads – Applications in Biotechnology and Medicine* (Geneva, 31 August–4 September, 1986).

ODERA, M., TAKEUCHI, K. & TOH-E, A. (1986). Molecular cloning of lipase genes from *Alcaligenes denitrificans* and their expression in *Escherichia coli*. *Journal of Fermentation Technology* **64**, 363–371.

OHKAWA, I., SHIGA, S. & KAGEYAMA, M. (1979). An esterase on the outer membrane of *Pseudomonas aeruginosa* for the hydrolysis of long chain acyl esters. *Journal of Biochemistry* **86**, 643–656.

OLSEN, R. H., DEBUSSCHER, G. & MCCOMBIE, W. R. (1982). Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *Journal of Bacteriology* **150**, 60–69.

ORNSTON, L. N. & STANIER, R. Y. (1966). The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. *Journal of Biological Chemistry* **241**, 3776–3786.

ROYLE, P. L., MATSUMOTO, H. & HOLLOWAY, B. W. (1981). Genetic circularity of the *Pseudomonas aeruginosa* PAO chromosome. *Journal of Bacteriology* **145**, 145–155.

STANISICH, V. A. & HOLLOWAY, B. W. (1972). A mutant sex factor of *Pseudomonas aeruginosa*. *Genetical Research* **19**, 91–108.

STUER, W., JAEGER, K.-E. & WINKLER, U. K. (1986). Purification of extracellular lipase from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **168**, 1070–1074.

TSAI, C.-M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.

ULITZUR, S. (1979). A sensitive bioassay for lipase using bacterial bioluminescence. *Biochimica et biophysica acta* **572**, 211–217.

WINGENDER, J. & WINKLER, U. K. (1984). A novel biological function of alginate in *Pseudomonas aeruginosa* and its mucoid mutants: stimulation of exolipase. *FEMS Microbiology Letters* **21**, 63–69.

WINKLER, U. K. & STUCKMANN, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. *Journal of Bacteriology* **138**, 663–670.

WINKLER, U., WINGENDER, J. & JAEGER, K.-E. (1985). Infektionen der Atemwege mit *Pseudomonas aeruginosa* bei der Cystischen Fibrose. *Klinische Wochenschrift* **63**, 490–498.

WRETLIND, B. & PAVLOVSKIS, O. R. (1984). Genetic mapping and characterization of *Pseudomonas aeruginosa* mutants defective in the formation of extracellular proteins. *Journal of Bacteriology* **158**, 801–808.

YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.