Kinetics of secretion of recombinant acid phosphatase by *Myxococcus xanthus*: a sensitive probe for the assay of protein translocation through the envelopes

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The gene encoding a periplasmic pH 2.5 acid phosphatase (*appA*) from *Escherichia coli* was placed under the control of an inducible promoter and integrated into the chromosome of *Myxococcus xanthus*. The majority of the AppA protein synthesized in *M. xanthus* accumulated in the periplasm whereas about 30% was secreted into the medium. The kinetics of AppA secretion through the 'outer envelopes' (i.e. periplasm + outer membrane) were followed after AppA induction. The results suggest that AppA crosses these envelopes by a mechanism involving diffusion and show that the periplasmic accumulation of AppA and envelope permeability are decreased by mutations that decrease the secretion of native proteins in *M. xanthus*.

Introduction

Myxococcus xanthus is a Gram-negative scavenging bacterium that belongs to the myxobacteria. To feed upon other micro-organisms, these bacteria secrete numerous hydrolytic enzymes during exponential growth (Rosenberg & Varon, 1984), and also during a multicellular developmental cycle characteristic of this family that leads to the formation of fruiting bodies (Shimkets, 1989). Under laboratory growth conditions, up to 50 different secreted proteins have been distinguished by electrofocusing of the culture medium of strain DK101 (Nicaud *et al.*, 1984).

Previous studies have shown that the secretory mechanism in *M. xanthus* does not have the same high degree of specificity as the secretory systems found in other Gram-negative species (for review see Pugsley, 1988, 1993; Hirst & Welch, 1988; Letouvet-Pawlak, 1991*b*) with the exception of the leakage process induced by the colicin lysis protein in *Escherichia coli* (Baty *et al.*, 1987). For example, *Erwinia chrysanthemi* pectate lyases can be secreted from a recombinant *M. xanthus* strain (Breton *et al.*, 1986), although they are not secreted by a recombinant strain of a closely related species, *Erwinia carotovora* subsp. *carotovora* (He *et al.*, 1991). Fur-

thermore, the TEM1 β -lactamase as well as the *E. coli* pH 2.5 acid phosphatase, both of which are normally periplasmic enzymes, are partially released from recombinant *M. xanthus* (Breton & Guespin-Michel, 1987). Nonetheless, it is presumed that protein secretion in *M. xanthus* occurs by a two-step mechanism involving export of proteins into the periplasm, followed by their secretion through the outer membrane. The foreign proteins that have been expressed and secreted by *M. xanthus* are all synthesized as precursors with a signal peptide (Breton *et al.*, 1986; Breton & Guespin-Michel, 1987). One additional argument in favour of the requirement for a signal peptide in protein secretion by *M. xanthus* proceeds from the fact that *TnphoA* (Manoil & Beckwith, 1985) random insertions yield chimeric proteins displaying phosphatase activity, some of which are extracellular (Kalos & Zissler, 1990; Breton *et al.*, 1990).

On the other hand, transposon insertion mutants, designated Exc[±], have been obtained (Nicaud *et al.*, 1984) that display a decrease in the amount of most extracellular proteins, as well as slime (Masson & Guespin-Michel, 1988), with no evidence of protein accumulation in the periplasm, in contrast to what was shown in *Pseudomonas aeruginosa* (Wretling & Pavlovskis, 1984), *Aeromonas hydrophila* (Howard & Buckley, 1983) or *E. chrysanthemi* (Andro *et al.*, 1984; Thurn & Chatterjee, 1985).

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These $\text{Exc}^{\pm}$ mutants display several unusual properties: (i) most of the extracellular enzymic activities assayed thus far are decreased, as is the total amount of secreted protein, but the protein patterns obtained by electrofocusing culture supernatants differ only slightly from those of the parental Exc^+ strain (Nicaud *et al.*, 1984); (ii) mutations leading to this phenotype belong to several unlinked loci and prevent fruiting body formation, which suggests a relationship between protein secretion and development (Petit *et al.*, 1993); (iii) the production of foreign pH 2.5 acid phosphatase is also impaired by $\text{Exc}^{\pm}$ mutations which decrease both cell-bound and extracellular activities (Breton & Guespin-Michel, 1987). This shows that these $\text{Exc}^{\pm}$ mutations impair genes that regulate the production of extracellular proteins at a post-transcriptional level, for instance at the level of export or secretion. However, a more precise analysis of the two steps involved in protein secretion, i.e. export and outer envelope translocation, is necessary.

To carry out this analysis, we took advantage of the fact that *E. coli* pH 2.5 acid phosphatase is only partly secreted [whereas most native proteins have not been detected in the periplasm (Nicaud *et al.*, 1984)]. The *appA* gene that encodes this protein was cloned under the control of the IPTG-inducible *tac* promoter and introduced in the chromosome of different strains of *M. xanthus*.

We showed that secretion of the AppA protein by *M. xanthus* can be quantified by two parameters that reflect the permeability of the outer envelopes toward this protein and that an $\text{Exc}^{\pm}$ mutation yields both a decrease in outer membrane permeability and a decrease in the quantity of the foreign protein in the periplasm.

Methods

Bacterial strains, plasmids and phages. These are listed in Table 1.

Media. *M. xanthus* was grown in CTT medium at 30 °C (Bretscher & Kaiser, 1978). *E. coli* was grown in LB medium (Miller, 1972) at 37 °C. Media were solidified with 1.2% (w/v) agar when necessary.

Plasmid construction and cloning. T4 DNA ligase and restriction enzymes were obtained from Boehringer Mannheim. All enzymes were used according to the specifications of the manufacturer. The ligation was performed overnight at 16 °C. Plasmid DNA was isolated from host bacteria as described by Holmes & Quigley (1981) for plasmids smaller than 15 kb, and according to Birnboim & Doly (1979) for plasmids larger than 15 kb. Transformations were performed using calcium chloride as described by Dagert & Ehrlich (1974). Cells used in transformations (*E. coli* strain DH5 α MCR) were diluted into 1 ml LB medium after heat shock and grown for 1 h at 37 °C before being plated on to selective medium containing ampicillin (500 $\mu\text{g ml}^{-1}$).

Construction of a cointegrate for *appA* transfer into *M. xanthus*. pME472 is a conjugative IncP plasmid in which two tandemly repeated IS21 elements allow the formation of cointegrates with other plasmids. pGM589 is a cointegrate obtained according to Breton & Guespin-Michel (1987), between pME472 and the recombinant plasmid

pGM588, which can be transferred by conjugation from the *E. coli* donor to *M. xanthus*.

***In vivo* gene transfer procedures.**

(i) **Matings.** Filter matings were performed overnight as described previously (Breton *et al.*, 1985). *M. xanthus* transconjugants were selected for their resistance to kanamycin (75 $\mu\text{g ml}^{-1}$).

(ii) **Transduction of Tn5 insertion Ω 031 from strain CM031 to strain CM243.** Strain CM243 was transduced with Mx4^{ts} lysate grown on CM031 according to Campos *et al.* (1978). The transductants were selected by the Tn5-borne resistance to phleomycin (Saulnier *et al.*, 1988). The transductants were $\text{Exc}^{\pm}$ and Fru^- , and had retained the other characteristics of CM243. Clone CM245 was retained.

pH 2.5 acid phosphatase detection on plates. The method was developed for *E. coli* by Boquet *et al.* (1987): the pH 2.5 acid phosphatase (Dassa & Boquet, 1985) activity was revealed by using *p*-nitrophenyl phosphate (PNPP) as substrate at 37 °C, pH 2.5. Yellow haloes of *p*-nitrophenol (PNP) surround the App⁺ clones.

The above method was adapted for use in *M. xanthus* by Breton & Guespin-Michel (1987). The App⁺ clones displayed a yellow colour which could only be seen on tan colonies.

Enzyme assays. Cells and supernatant were separated by centrifugation for 5 min in a microfuge.

(i) **pH 2.5 acid phosphatase.** The assays were performed according to Dassa & Boquet (1981) with PNPP as substrate. One unit of enzyme

Table 1. *Strains, plasmids and phages*

Relevant characteristics		References
Strains		
<i>E. coli</i>		
DH5 α MCR	<i>recA1 mcrA mcrB hsdR endA1</i>	Hanahan (1983)
W3101 Nal ^r	<i>recA1 Try⁻ Nal^r</i>	Kopecko <i>et al.</i> (1976)
C600	<i>thi1 thr1 leuB6 lacY1 tonA1 supE44</i>	Appleyard (1954)
<i>M. xanthus</i>		
DK1622	Wild type, yellow	Kaiser (1979)
DK101	Yellow, Fru ⁺ , Mot [±] , Exc ⁺	Hodgkin & Kaiser (1979)
DZ1	Tan, Fru ⁻ , Mot ⁻ , Exc [±] , Str ^r	Zusman <i>et al.</i> (1978)
CM240	DK1622 Str ^r	This work
CM031	DK101::Tn5 Exc [±] Fru ⁻ Tan	Nicaud <i>et al.</i> (1984)
CM2214	DZ1::pGM589	This work
CM2217	DZ1::pGM589*	This work
CM2216	DZ1::(<i>appA lacZ Km^r</i>)	Letouvet-Pawlak (1991a)
CM243	CM240::pGM589*	This work
CM245	CM243::Tn5 (Ω 031)	This work
Plasmids		
pTTQ18	Ap ^r , <i>lacI^q</i> , <i>p^{tac}</i> , polylinker	Stark (1987)
pME472	IncP, Km ^r , Tc ^r , Ap ^r , 2 IS21	Reimann <i>et al.</i> (1988)
pGM588	pTTQ18:: <i>appA</i>	This work
pGM589	pGM588::pME472	This work
pGM589*	pGM589 retransferred from GM589 (HFI)	This work
pPB1132	pBR322:: <i>appA</i> Tc ^r , Ap ^r ,	Boquet <i>et al.</i> (1987)
Bacteriophages		
Mx4 ^{ts} hrm	Transduction phage from <i>M. xanthus</i>	Campos <i>et al.</i> (1978)

Fig. 1. (a) Construction of plasmid pGM588. The *Stu*I, *Bgl*II, *Sma*I and *Bam*HI digestions and the insert-vector ligation were performed as described in Methods. The *Stu*I and *Sma*I restriction enzymes both generate blunt ends, and the *Bgl*II and *Bam*HI restriction enzymes

corresponds to the amount of enzyme which produces 1 nmol PNP min^{-1} at 37 °C, pH 2.5. The molar extinction coefficient of PNP, measured under the assay conditions, is $1.78 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 410 nm (the spontaneous hydrolysis of PNPP was routinely assayed and subtracted from our values).

(ii) *β -Galactosidase*. The assays were performed on cells and supernatant by the procedure of Miller (1972) using *O*-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate. One unit of β -galactosidase was defined as the amount of enzyme producing 1 nmol *O*-nitrophenol (ONP) min^{-1} at 28 °C, pH 7.

(iii) *Proteolytic activity*. The total proteolytic activity was assayed in the supernatant at pH 9.6 with azocasein as substrate (Millet, 1970).

Cell fractionation in M. xanthus. We used the cell fractionation procedure of Hartzell & Kaiser (1991) with some modifications. Liquid-grown cultures of the *M. xanthus* CM2216, a recombinant strain producing both β -galactosidase (used as cytoplasmic marker) and pH 2.5 acid phosphatase, were harvested at a cell density of 4×10^8 cells ml^{-1} and centrifuged at 8000 *g* for 10 min at 15 °C. The cells were washed in 1 vol. cold 50 mM-Tris (pH 7.5) and centrifuged at 16000 *g* for 15 min at 5 °C. The supernatant was designated the 'Tris fraction'. The pellet was suspended in 0.25 vols cold 0.75 M-sucrose/10 mM-Tris-acetate (pH 7.9). Lysozyme and EDTA were added to final concentrations of 2 mg ml^{-1} and 0.5 mM, respectively. The reaction was incubated on ice for at least 1 h and then centrifuged at 21000 *g* for 10 min at 5 °C. The supernatant, containing part of the periplasm, was retained and named the 'PI fraction'. For the enzymic assays, a dilution of the PI fraction was required because EDTA inhibits a large part of the β -galactosidase activity and, to a lesser extent, the acid phosphatase activity. The pellet was suspended in 2 vols cold 0.1 M-sucrose/10 mM-Tris-acetate (pH 7.9) and incubated on ice. After 2 h, most of the cells had formed spheroplasts. However 5–6 % of the cells in the suspension were still intact bacilli as estimated by microscopic examination. The preparation was centrifuged at 16000 *g* for 10 min at 5 °C. The supernatant contained the remainder of the periplasm (PII fraction). The pellet was suspended in 1 vol. cold distilled water and incubated on ice for at least 30 min. To obtain a complete lysis of the spheroplasts, it was often necessary to subject the spheroplasted cells to sonication or freeze-thawing.

Results

Construction of *M. xanthus* strain bearing the *appA* gene under the control of the *tac* promoter

The *appA* gene encoding *E. coli* pH 2.5 acid phosphatase was cloned into the polylinker of plasmid pTTQ18 to generate plasmid pGM588 (Fig. 1a); plasmid pTTQ18 contains the *lacI*^q *p*^{tac} system and a polylinker region located downstream of *p*^{tac} (Stark, 1987). The *E. coli* strain DH5 α MCR was transformed with pGM588, and one of the transformants, screened on plate for IPTG-inducible pH 2.5 acid phosphatase activity, was retained. The method of Breton & Guespin-Michel (1987) was used to integrate plasmid pGM588 into the chromosome

generate compatible cohesive ends. (b) Map of plasmids pGM589 and pGM589*. The asterisk represents a fragment of 6.1 kb in pGM589 and 5.8 kb in pGM589*.

Fig. 2. Localization of β -galactosidase and pH 2.5 acid phosphatase in the different cell compartments of *M. xanthus* strain CM2216. Cells were grown in CTT medium supplemented with kanamycin ($75 \mu\text{g ml}^{-1}$). The enzyme assays were performed as described in Methods. (a) Extracellular (▨) and cell-bound (■) activities. Supernatant and cells were separated by centrifugation in a microfuge for 5 min. (b) Distribution of the activities during the different steps of the fractionation procedure. Cell-bound β -galactosidase activity (■); cell-bound pH 2.5 acid phosphatase activity (□). Cells were fractionated by the procedure described in Methods. The Tris fraction was the washing supernatant; PI fraction containing part of the periplasm was obtained after treatment of the cells by lysozyme-EDTA; PII fraction was the result of a slight osmotic shock which released the remainder of the periplasm; and cytoplasmic fraction was from lysed spheroplasts.

of *M. xanthus*, using a cointegrate between plasmid pGM588 and plasmid pME472 as described in Methods. The resulting cointegrate obtained, pGM589 (Fig. 1b), was transferred by conjugation from *E. coli* to *M. xanthus* strain DZ1. This strain was chosen because it is the best recipient for conjugative plasmids (Breton *et al.*, 1985), it produces low enough amounts of extracellular proteases not to decrease the secreted pH 2.5 acid phosphatase (result not shown), and preliminary studies on the secretion of this foreign protein have been performed with this strain (Breton & Guespin-Michel, 1987). However, it is a pleiotropic, non-motile, fruitless mutant and it resembles *Exc*[±] mutants in its ability to secrete only half the total amount of extracellular proteins secreted by the wild type (Masson & Guespin-Michel, 1988).

One *M. xanthus* transconjugant, designated CM2214, that displayed IPTG-inducibility of *appA* gene expres-

sion, was retained for further studies. The strain was maintained under selective pressure with carbenicillin to stabilize the inserted plasmid pGM588 and oxytetracycline to stabilize the cointegrate (Jaoua *et al.*, 1986). In addition, the number of cells retaining the ability to produce the pH 2.5 acid phosphatase was monitored during each experiment.

Lysis control

We first checked that secretion was independent of lysis by measuring the appearance in the extracellular medium of both β -galactosidase and pH 2.5 acid phosphatase when a *M. xanthus* recombinant strain CM2216 (Letouvet-Pawlak, 1991a) harbouring both the *E. coli lacZ* and *appA* genes under the control of the same constitutive promoter, was grown in CTT medium containing kanamycin ($75 \mu\text{g ml}^{-1}$). In this strain, the total specific activity of β -galactosidase was $152 \pm 2 \text{ U (mg cell protein)}^{-1}$ and the total specific activity of the acid phosphatase was $148 \pm 3 \text{ U (mg cell protein)}^{-1}$. As seen in Fig. 2(a), 20% of the acid phosphatase but only 2% of the β -galactosidase were found in the culture supernatant.

Localization of the cell-bound AppA protein

This protein is located in the periplasm of its natural host *E. coli* and is processed from a 47 kDa precursor by cleavage of a 2.4 kDa N-terminal signal peptide (Dassa *et al.*, 1990). Localization of the protein in *M. xanthus* was achieved by separation of the different cell compartments by the method of Hartzell & Kaiser (1991), with the cytoplasmic marker protein β -galactosidase being used as a control for lysis during cell fractionation. For this purpose, we had to utilize the doubly recombinant strain CM2216 in spite of its enhanced fragility. Protease inhibitors were not required since CM2216 secretes less protease activity than wild type *M. xanthus* strains.

Approximately 80% of the AppA activity was released with the periplasmic fraction (Fig. 2b). To avoid lysis of the spheroplasts during the preparation of the periplasmic fraction, we resuspended the pellet after the lysozyme-EDTA treatment in 0.1 M-sucrose.

The Tris fraction (washing supernatant) contained 2.1% of both activities indicating that only a small fraction of the cells had lysed at this stage (Fig. 2b).

β -Galactosidase activity assayed in the total periplasmic fraction (PI + PII; see Methods) was very low (1.5% of the cell-bound activity), while 79.4% of the pH 2.5 acid phosphatase cell-bound activity was present in this total periplasmic fraction (Fig. 2b). In contrast, 96.4% of the β -galactosidase activity and only 18.5% of the phosphatase activity were detected in the cytoplasmic fraction (obtained by suspending sphero-

Fig. 3. pH 2.5 acid phosphatase production in cells (a) and supernatant (b) in *M. xanthus* strain CM2214, after IPTG induction during mid-exponential phase. The pH 2.5 acid phosphatase activity is expressed per mg cell protein. Cells were grown in CTT medium supplemented with oxytetracycline ($10 \mu\text{g ml}^{-1}$) and carbenicillin ($250 \mu\text{g ml}^{-1}$) to ensure selective pressure of the cointegrate. IPTG was added at time 0 at final concentrations of 10^{-3} M ($\square$), 2×10^{-5} M ($\blacksquare$), or 10^{-5} M ($\triangle$). The pH 2.5 acid phosphatase assays were performed as described in Methods. (a) The activity in uninduced cells ($\bullet$) was $15.1 \text{ U AppA (mg cell protein)}^{-1}$. (b) The activity in the supernatant of uninduced cells ($\bullet$) was $5.4 \text{ U AppA (mg cell protein)}^{-1}$.

plasts in cold water) (Fig. 2b). The latter figure is over estimated, however, since the cytoplasmic fraction is contaminated with 5 to 6 % of intact cells. Therefore, we conclude that the cell-bound acid phosphatase activity is located in the periplasm.

Kinetics of AppA accumulation in the periplasm and extracellular medium of *M. xanthus* DZ1 recombinant strain CM2214

Exponentially growing cells of strain CM2214 were supplemented by IPTG, and pH 2.5 acid phosphatase was assayed in the cells (Fig. 3a) and supernatant (Fig. 3b) at different times after induction. The means of repeated experiments are reported in Table 2.

Previous studies on the *lacI^q p^{tac}-lacZ* system in *M. xanthus* had shown that the level of induction increased in a sigmoidal fashion with IPTG concentration (Letouvet-Pawlak *et al.*, 1990); accordingly, we used three different IPTG concentrations: 10^{-3} M, 2×10^{-5} M and 10^{-5} M.

Table 2. Distribution of pH 2.5 acid phosphatase activity in the cells and enzyme accumulation in the supernatant after induction of *M. xanthus* strain CM2214 with different concentrations of IPTG

Results are the mean of 3–5 experiments. The slope of the accumulation curve was estimated by linear regression (r = correlation coefficient). These results take into account the instability of strain CM2214: we used a correction coefficient calculated by comparison of the levels of activity in uninduced cells obtained in the different experiments.

IPTG concn (M)	pH 2.5 acid phosphatase activity in the recombinant cells [$\text{U (mg cell protein)}^{-1}$]	Slope of the accumulation of acid phosphatase in the supernatant as a function of time [$\text{U (mg cell protein)}^{-1} \text{ h}^{-1}$]
10^{-3}	71.1 ± 12	0.7 ± 0.15 ($r \geq 0.986$)
2×10^{-5}	42.9 ± 3.5	0.35 ± 0.05 ($r \geq 0.964$)
10^{-5}	29.8 ± 3	0.23 ± 0.02 ($r \geq 0.926$)
0	14.6 ± 1	–

Cell-bound pH 2.5 acid phosphatase accumulation was very similar to that obtained with β -galactosidase when the *lacZ* gene was expressed in *M. xanthus* under the control of *p^{tac}* (Letouvet-Pawlak *et al.*, 1990). The increase of the specific activity over the uninduced background was dependent on IPTG concentration; pH 2.5 acid phosphatase started accumulating in the cells less than 1 h after induction with 10^{-3} M and 2×10^{-5} M-IPTG, and 2 h after induction with 10^{-5} M-IPTG. No decrease of specific activity was found until 53 h after induction.

pH 2.5 acid phosphatase secretion (Fig. 3b) is expressed as the ratio of the extracellular activity to the cell protein content. Three phases can be described: phase 1 corresponds to a retention of the enzyme in the cells; phase 2 corresponds to a linear increase of the extracellular phosphatase activity per mg of cell protein, the slope of which is roughly proportional to the maximal specific activity of the cell-bound enzyme (Table 2); the third phase is reached 30–45 h after IPTG induction and corresponds to a steady state: the extracellular activity is then equal to $28 \pm 2\%$ of the total activity. The proteolytic activity produced in the medium was assayed. No difference could be observed with or without IPTG, suggesting that induction of pH 2.5 acid phosphatase production and secretion of the enzyme did not disturb the overall secretory mechanism.

Probing secretion in other strains

New strains were constructed in a continuation of the study. A mating was performed between strain CM2214

Fig. 4. pH 2.5 acid phosphatase production in cells (a) and supernatant of cultures (b) of *M. xanthus* strain CM2217 after resuspension of growing cells in fresh CTT medium. The resuspension medium was supplemented with oxytetracycline as the culture medium. The pH 2.5 acid phosphatase activity is expressed per mg cell protein. This activity was assayed as described in Methods. (a) Accumulation of cell-bound specific activity in non-induced cells resuspended in CTT plus 10^{-3} M-IPTG. (b) Activity in the supernatant. ($\square$) Non-induced cells resuspended in CTT minus IPTG; ($\blacktriangle$) non-induced cells resuspended in CTT plus 10^{-3} M-IPTG; ($\blacksquare$) induced cells resuspended in CTT plus 10^{-3} M-IPTG (i.e. in fully induced conditions).

(DZ1::pGM589) as donor and *E. coli* W3101 Nal^r as recipient. Jaoua *et al.* (1990) have shown that the integration of the IncP plasmid RP4 (Datta *et al.*, 1971) in the chromosome of *M. xanthus* is followed by different DNA rearrangements. Modified plasmids, called RP4*, may be obtained upon conjugation with *E. coli* and display a higher frequency of integration when transferred back to *M. xanthus* (HFI plasmids). This proved to hold true with RP4 derivatives such as the cointegrate pGM589, since the retransferred cointegrate pGM589* was shown to possess all the characteristics of pGM589, but was transferable to *M. xanthus* strains with a much higher frequency (1.5×10^{-5} when the recipient strain was DK101 instead of 10^{-7} for pGM589) and was more stably maintained. A deletion of 0.3 kb was observed in the 6.1 kb *Pst*I restriction fragment of pME472 which was similar to that previously found in HFI plasmids (Jaoua *et al.*, 1990) (Fig. 1b). CM2217 and CM243 are recombinants producing IPTG-inducible acid phosphatase obtained by transferring plasmid pGM589* into strain DZ1 and strain CM240 (Exc⁺), respectively.

Fig. 5. pH 2.5 acid phosphatase production in cells (a) and supernatant of cultures (b) of *M. xanthus* strain CM243 after resuspension of growing cells in fresh CTT medium. Conditions and symbols are as described in the legend to Fig. 4. (a) Accumulation of cell-bound specific activity in non-induced cells resuspended in CTT plus 10^{-3} M-IPTG. (b) Activity in the supernatant.

The experiment described above presents the major drawback that acid phosphatase activity decreases in the supernatant of strains producing wild type levels of proteases. We therefore modified the experimental procedure in order to study AppA secretion under conditions where proteolysis was negligible in all strains. This was achieved by pelleting growing cells and suspending them in fresh CTT medium (devoid of proteases), thus allowing AppA protein secretion to be followed under non-induced conditions (NI), fully induced conditions (I), or when induction was started concomitant with the resuspension.

When these experimental conditions were tested on strain CM2217 (DZ1::pGM589*) it was found that kinetics of appearance of extracellular AppA and cell-associated AppA activity differed upon induction with IPTG. For cells previously induced with IPTG and resuspended in fresh CTT medium containing IPTG, AppA activity in the medium was detected immediately and increased in a linear fashion with the slope of the curve being proportional to the amount of cell-bound AppA present in the cells at the time of resuspension (Fig. 4b). However, when non-induced cells were resuspended in IPTG-supplemented medium the level of AppA secretion remained identical to that of the non-

Table 3. Comparison of secretion characteristics of three recombinant strains

	CM2217	CM243	CM245
Cell-bound AppA specific activity in non-induced cells [U (mg cell protein) ⁻¹]	14 ± 1	14 ± 4	8.6 ± 1.5
Cell-bound AppA specific activity in fully induced cells [U (mg cell protein) ⁻¹]	88.7 ± 10	30 ± 5	17.7 ± 5
Slope of accumulation of AppA in the supernatant as a function of time [U (mg cell protein) ⁻¹ h ⁻¹]			
NI (-)*	0.28 ± 0.02	0.7 ± 0.1	0.33
I (+)	1.6 ± 0.2	2 ± 0.2	0.73 ± 0.03
Relative secretion rate (h ⁻¹)†			
NI (-)	2 ± 0.2	5 ± 2	3.8 ± 1
I (+)	1.8 ± 0.2	6.6 ± 2	4.2 ± 1.5
Retention time (h)‡	5–6	< 1	5–6

* NI, Non-induced cells; I, induced cells; (-), resuspension in CTT; (+), resuspension in CTT plus 10⁻³ M-IPTG.

† The relative rate of secretion is the ratio 'slope of the secretion curve of AppA/AppA specific activity in the resuspended cells × 100'.

‡ The retention time is the time during which the slope of the secretion curve obtained upon IPTG induction is identical to that obtained in non-induced conditions.

induced cells for about 5 h, and then increased (Fig. 4b), in contrast to the level of periplasmic AppA activity which increased 1 h after resuspension (Fig. 4a). This defines a retention time of about 5 h, as previously demonstrated, as well as a linear increase of extracellular AppA production.

When the same experiment was performed with strain CM243 (Fig. 5), the overall kinetics were similar. AppA accumulation in cells after induction started to occur in less than 1 h and reached a plateau corresponding to the fully induced level. The curves of extracellular AppA secretion were linear and their slopes were proportional to the periplasmic AppA concentration at the time of resuspension. However several differences between strains CM243 and CM2217 were observed (Table 3): (i) the levels of cell-bound activities were different; (ii) the relative secretion rate, defined as the ratio 'slope of secretion curve/periplasmic AppA specific activity' – a constant for induced and non-induced conditions, was higher in strain CM243; (iii) the retention time was less than 1 h in strain CM243. The significance of these discrepancies is not clear however, since the parental strains DK1622 and DZ1 may differ in several characteristics, and the location of the inserted pGM589* may also be different in strains CM2217 and CM243 (Breton *et al.*, 1985; Breton & Guespin-Michel, 1987; Jaoua *et al.*, 1990). A significant comparison between the phenotypes Exc⁺ and Exc[±] requires isogenic strains that

Fig. 6. pH 2.5 acid phosphatase production in cells (a) and supernatant of cultures (b) of *M. xanthus* strain CM245 after resuspension of growing cells in fresh CTT medium. Conditions and symbols are as described in the legend to Fig. 4. (a) Accumulation of cell-bound specific activity in non-induced cells resuspended in CTT plus 10⁻³ M-IPTG. (b) Activity in the supernatant.

differ only in their overall ability to secrete native proteins.

Strain CM031 is an Exc[±] Tn5 insertion mutant of strain DK101 (Nicaud *et al.*, 1984). Petit *et al.* (1993) showed that the Exc[±] phenotype can be transduced along with the Tn5 insertion (Ω031) into strain DK1622. Similarly, strain CM245 is an Exc[±] CM243::Tn5 Ω031 strain constructed by Mx4^{ts} transduction. Fig. 6 shows that strain CM245 displays an AppA protein secretion pattern different from that obtained with the parental strain CM243, but rather similar to that obtained with strain CM2217, i.e. a retention time of 5–6 h (although cell-bound accumulation begins less than 1 h after induction) and a slope of the secretion curve that is less steep than that obtained with CM243. In addition, the total production of AppA was nearly twofold lower in CM245 compared with CM243 for both induced and non-induced cultures.

Table 3 summarizes these results. Two parameters may be used to characterize AppA protein secretion: the retention time which is much shorter in the Exc⁺ strain, and the relative rate of secretion which is independent of the specific activity of the cells, different in all three strains, and higher in the Exc⁺ strain. The cell-bound specific activity in both induced and non-induced cells is also a relevant parameter since it is reduced in the Exc[±]

CM245 recombinant relative to the Exc^+ parental strain CM243.

Discussion

The fact that the *E. coli* periplasmic pH 2.5 acid phosphatase, when expressed in *M. xanthus* from its gene inserted into the host chromosome, is partially secreted and partially cell-bound, in contrast to native extracellular proteins that are fully secreted, has enabled us to construct a probe to analyse the two steps of the protein secretion mechanism in *M. xanthus*.

We have shown that this secretion is not due to cell lysis and that the cell-bound acid phosphatase is mainly, if not totally, located in the periplasm of the recombinant *M. xanthus* cells. Therefore the production of extracellular AppA reflects the translocation of the protein through the outer envelope.

We made use of these properties to construct a probe consisting of a transferable cointegrate able to be inserted at random in the *M. xanthus* chromosome, in which the *appA* gene is under the control of the inducible *tac* promoter. This construct was first inserted in strain DZ1. Extracellular AppA is not degraded in the culture supernatant from this strain, and the kinetics of production and secretion of this protein can be followed over several generations until the steady state is reached.

The kinetics of export of acid phosphatase are more rapid than those of secretion, which is characterized by an interval during which the protein accumulated in the periplasm is not released into the medium (the retention time) and by the fact that extracellular AppA accumulation expressed per mg of cell protein increases linearly (for 30–45 h) with a slope that depends on the amount of cell-bound phosphatase.

These results could be explained if protein diffusion through the periplasmic gel and/or the outer membrane was the limiting step of the secretion process. The initial stage of enzyme retention in the cells preceding secretion would therefore correspond to the time required for its diffusion through one and/or the other of these structures, and the percentage of enzyme released into the extracellular medium would depend on its diffusion coefficient.

The existence of a periplasmic gel has been proposed by Kellenberger (1990); thus, in *M. xanthus*, the proteins could diffuse in this gel with a specific diffusion coefficient dependent on their size, conformation and charge, to the inner side of the outer membrane, before crossing this membrane. Diffusion could also be the translocation mechanism through the outer membrane. Baty *et al.* (1987) proposed that colicin A could reach the extracellular medium in *E. coli* by slow diffusion of the protein through the inner and outer membranes, after an increase

in the permeability of these membranes due to the action of the lysis protein.

When the kinetics were studied for a shorter time with cells resuspended in fresh CTT, essentially the same results are obtained. The slopes of the curve of accumulation of extracellular AppA reflect the secretion rates; thus, the ratio 'slope of the curve/periplasmic AppA specific activity' was referred to as the 'relative rate of secretion' which is independent of the cell-bound specific activity.

Can results obtained with a foreign protein, chosen because it was secreted less efficiently by *M. xanthus* than the native proteins, be of biological significance with regard to the mechanism of secretion of these native proteins? It is comparison of results obtained with CM243 and CM245, isogenic recombinant strains that differ by a single $\text{Exc}^\pm$ mutation, which gives credit to the method, since this mutation impairs the secretion of most native proteins (Petit *et al.*, 1993).

The relative rate of secretion is higher in the Exc^+ recombinant. In this strain the retention time, if any, is less than 1 h, whereas it is 5–6 h in the $\text{Exc}^\pm$ mutant and also in strain DZ1 which behaves as an $\text{Exc}^\pm$ mutant. Thus, the $\text{Exc}^\pm$ mutation introduced in the wild type recombinant modifies the permeability properties of the envelopes toward AppA protein. In addition, this mutation also decreases the amount of accumulated periplasmic AppA. This may be due to increased degradation of this protein during the long retention time, or it may reflect a coupling between export and secretion, which would represent another peculiarity of the secretory mechanism in *M. xanthus*.

Since the overall effect of the $\text{Exc}^\pm$ mutation on native protein secretion is the same as that observed with protein AppA, it is likely that native proteins are secreted by the same route as this foreign protein, although their structure would allow a more efficient diffusion.

Regardless of the actual mechanism, our method has allowed the definition of two parameters (retention time and relative secretion rate of the AppA protein) that characterize the permeability and hence the structure of the envelopes of different strains of *M. xanthus*. These parameters should aid in the analysis of different $\text{Exc}^\pm$ mutations (which are also unable to undergo development in response to nutrient starvation) and the monitoring of possible changes in membrane permeability during development in the wild type strain.

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