β -Galactosidase and Alkaline Phosphatase Do Not Become Extracellular When Fused to the Amino-terminal Part of Colicin N

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A series of plasmids encoding hybrid proteins comprising various lengths of the NH₂-terminal region of colicin N coupled to almost complete β -galactosidase or alkaline phosphatase polypeptides was constructed by transposon mutagenesis of ColN plasmid derivatives. Synthesis of the hybrid proteins, like that of colicin N itself, was regulated by the SOS response. Large quantities of the hybrid proteins accumulated in the cytoplasm (β -galactosidase) or particulate fractions (alkaline phosphatase). When the gene fusions were expressed in cells that were producing colicin E2 and expressing the ColE2 lysis gene, only very low levels of the hybrid proteins were found in the medium. The results suggest that the amino-terminal part of colicin N does not contain sufficient biochemical information to promote the release of the hybrid proteins into the medium.

INTRODUCTION

An increasing awareness that many different Gram-negative bacteria can secrete proteins into the growth medium, coupled with the potential use of protein secretion in biotechnology, has catalysed research into the ways in which polypeptides are translocated across the twin barriers created by the inner (cytoplasmic) and outer membranes (reviewed by Pugsley & Schwartz, 1985). The secretion of some proteins such as cholera toxin or pseudomonas exotoxin A (Hirst *et al.*, 1984; Lory *et al.*, 1983) seems to represent an extension of the signal peptide pathway by which Gram-negative bacteria export most of their periplasmic or outer membrane proteins. This is by no means always the case, however, as alternative routes are used in the secretion of haemolysin (Felmlee *et al.*, 1985) or colicins (Pugsley, 1984*b*) by *Escherichia coli* K12.

Colicins are encoded by plasmids, which, in most cases, carry a 'lysis' gene whose expression is essential for colicin efflux and which, like the colicin structural gene, is regulated by the SOS response (Oudega *et al.*, 1982; Pugsley & Schwartz, 1983; Jakes & Zinder, 1984; Lawrence & James, 1984; Pugsley, 1984*a*; Cavard *et al.*, 1985; Zhang *et al.*, 1985). The product of the lysis gene is a small envelope protein which stimulates an envelope phospholipase, probably to increase outer membrane permeability (Pugsley & Schwartz, 1984). The macropermeability of the inner membrane is not substantially affected by the activation of phospholipase (Pugsley & Rosenbusch, 1981) and the release of colicins from the cytoplasm, where they accumulate in large amounts (Cavard *et al.*, 1984), is not accompanied by the release of other cytoplasmic proteins. Specific regions of the colicin polypeptide might therefore be required to promote their release. However, lysis proteins encoded by different Col plasmids have highly conserved amino acid sequences (Cavard *et al.*, 1985; Chan *et al.*, 1985; Cole *et al.*, 1985; Hakkaart *et al.*, 1981; Watson *et al.*, 1984), and yet can promote the release of heterologous colicins (Pugsley &

Abbreviations: COLN-BGAL, colicin N- β -galactosidase hybrid polypeptide; COLN-PHOA, colicin N-alkaline phosphatase hybrid polypeptide.

Schwartz, 1983), which suggests that they may not interact directly with the colicins(s) to promote their transfer across the cytoplasmic membrane.

Previous studies in our laboratories showed that truncated colicins containing the amino-terminal 70% of the 39000 M_r colicin N or the amino-terminal 50% of the 62000 M_r colicin E2 were efficiently released from cells expressing the lysis gene (Pugsley, 1984a; Cole *et al.*, 1985). This suggested that any sequence required for release would be located in the NH_2 -part of the colicin polypeptides. This, in turn, raised the possibility of genetically fusing the NH_2 -terminal parts of these colicins to other polypeptides, thereby creating hybrid proteins which could be released from the cells upon expression of the lysis gene. Colicin N seemed particularly well suited for this purpose because it is smaller than most other colicins, and because Tn5 insertions in the colicin N structural gene, *cna*, lead to the production of stable, truncated colicin N polypeptides (Pugsley, 1984a). In this report, we describe the construction and analysis of hybrid genes containing varying lengths of *cna* coupled to *'lacZ*, which codes for the soluble cytoplasmic enzyme β -galactosidase, or to *'phoA*, which codes for a soluble, non-exported variant of the normally periplasmic alkaline phosphatase.

METHODS

Growth media. L broth and L broth agar (Miller, 1972) were used throughout. Antibiotics were used at the following concentrations ($\mu\text{g ml}^{-1}$): Ap, 200; Km, 50; Tc, 15. Mitomycin C was used at $0.5 \mu\text{g ml}^{-1}$ in broth cultures and at $0.1 \mu\text{g ml}^{-1}$ in agar media. In some cases, 20 mM-MgSO₄ was added to broth cultures to suppress partial lysis due to lysis gene expression. All cultures were grown aerobically at 37 °C.

Strains, plasmids and construction of gene fusions. The *E. coli* K12 strains and plasmids used in this study are listed in Table 1. The plasmids used to construct gene fusions were derived from pColN-284 (Pugsley, 1984a), or from pCHAP4, a ColN plasmid found in a new Col⁺ isolate from the River Rhine in Switzerland. pAPIP401, derived from pColN-284 (Pugsley, 1984a), and pCHAP12, derived from pCHAP4 (Fig. 1), were used for constructing gene fusions.

lacZ gene and operon fusions were obtained by miniMu d transposition as described by Castilho *et al.* (1984) using strains POII1681TR or POII1734TR (gene fusions) and POII1681TR or POII1734TR (operon fusions). pAPIP401 was introduced into these strains by transformation (Maniatis *et al.*, 1982) with selection for Ap^R. The transformants were then thermally induced to produce phage particles (Castilho *et al.*, 1984), which were used to infect strain PAP713 with selection for Ap^R Km^R on L agar plates. Randomly-selected colonies were tested for colicin production in patch tests (Pugsley, 1985) and for their LacZ phenotype on L agar + ampicillin + 5-bromo-4-indolyl β -D-galactoside (XGal) $\pm$ mitomycin C. Lac⁺ (blue) Col⁻ clones were retained for further analysis.

Alkaline phosphatase gene fusions were obtained by infecting strain PAPI03 (pCHAP12 pAPIP502) with TnphoA as described previously for Tn5 (Pugsley & Schwartz, 1983). Plasmids obtained by the method of Holmes & Quigley (Maniatis *et al.*, 1982) from independently-mutagenized cultures were used to transform strain PAP488 with selection for Ap^R Km^R on L agar + 5-bromo-4-chloro-3-indolyl phosphate. Transformants which produced blue (PhoA⁺) colonies were tested for colicin production in patch tests. Plasmid preparations from cultures of the

Table 1. *Escherichia coli* K12 strains and plasmids

Strain/plasmid	Characteristics	Source/reference
PAP103	$\Delta(lac-pro)$	Laboratory strain
PAP488	PAP103 <i>rpsL</i>	Laboratory strain
PAP713	<i>araD139</i> $\Delta(ara-leu)7679$ $\Delta(pro-arg-lac)$ <i>XIII ompF</i> Mu ⁺ <i>rpsL</i>	Laboratory strain*
BZB1011	<i>gyrA</i>	Laboratory strain
PAP308	BZB1011 <i>ompF</i>	Laboratory strain
PAP151	BZB1011 <i>rbs::Tn10 phoS</i>	Laboratory strain
pAPIP401	<i>ori</i> pBR322 <i>bla</i> ⁺ <i>cna</i> ⁺ <i>cni</i> ⁺ <i>cnl</i> ⁺	Pugsley (1984a)
pCHAP12	<i>ori</i> pUC9 <i>bla</i> ⁺ <i>cna</i> ⁺ <i>cni</i> ⁺ <i>cnl</i> ⁺	Fig. 1
pAPIP502	F ⁺ <i>pro</i> ⁺ $\Delta lacZM15$ <i>lacI</i> ^{q1} Tn10	Pugsley & Schwartz (1983)
pMM100	<i>ori</i> pACYC184 <i>tet</i> ⁺ <i>lacI</i> ^{q1}	M. Lavina†
pCH2	<i>ori</i> pBR322 <i>tet</i> ⁺ $\Phi(bla-phoA)$	Hoffman & Wright (1985)

* Derived from strain MM8820 (Castilho *et al.*, 1984).

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Col⁻ PhoA⁺ clones repeatedly gave heterogeneous patterns when examined by agarose gel electrophoresis (Maniatis *et al.*, 1982). This was apparently due to a high frequency of spontaneous deletion formation, which we overcame by deleting DNA between the *Bam*HI site within *TnphoA* and the *Bam*HI site at the junction between the pCHAP4 DNA and pUC9 DNA in pCHAP12 (Fig. 1). This removed IS50R, the probable cause of the deletions.

Plasmid analysis. Plasmids were digested with restriction endonucleases and ligated with T4 DNA ligase as described by Maniatis *et al.* (1982). Tris/acetate-buffered agarose gels containing 0.7–1.0% agarose were used for plasmid analysis (Maniatis *et al.*, 1982), and were calibrated with DNA fragments of known sizes.

Cell fractionation and protein analysis. Cell-free and cell-associated proteins were precipitated with TCA (Pugsley & Schwartz, 1984) and examined by SDS-PAGE essentially as described by Pugsley & Schnaitman (1979) using Tris/glycine buffers with 10% (w/v) acrylamide/0.13% bisacrylamide gels. Proteins were stained with Coomassie Blue. Cell-associated enzymes were released by sonication at 4 °C. Osmotic shock was effected as described by Nossal & Heppel (1966). Membrane fractions were obtained by centrifuging sonicated cells at 46000 *g* or 130000 *g* for 2 h. Enzymes were assayed according to Miller (1972; β -galactosidase), Brockman & Heppel (1968; alkaline phosphatase) or Raibaud *et al.* (1984; amylomaltase). Colicin was assayed as described previously (Pugsley & Rosenbusch, 1981).

RESULTS

Colicin *N*- β galactosidase fusion proteins

Fusions between *cna* and *lacZ* genes. These were obtained by mutagenizing plasmid pAPIP401 with miniMu dII1681 or miniMu dII1734 (Methods). Approximately 25% of the Ap^R Km^R transfectants were Col⁻ in patch tests and, of these, 15% produced pale or moderately blue colonies on LA plates containing XGal and dark blue colonies on the same medium containing 0.1 μ g mitomycin C ml⁻¹ to induce *cna* expression. This was close to the expected frequency (17%) for the random insertion of miniMu dII transposons giving in-frame *lacZ* gene fusions. The initial stages of selection were done in the absence of *cna* induction by mitomycin C. All resulting clones grew normally in L broth supplemented with 0.5 μ g mitomycin C ml⁻¹, indicating that expression of the *lacZ* gene fusions was not detrimental to cell viability.

A total of 11 independent Lac⁺ *cna-lacZ* gene fusion plasmids were further analysed. The location of the fusion site in pAPIP401 was determined by digestion with restriction endonucleases; the results in Table 2 show the positions of the insertion sites relative to co-ordinate 0, the calculated translation initiation site of *cna* (Fig. 1 and Pugsley, 1984a). Mitomycin C induction of strains carrying these plasmids resulted in the production of large amounts of presumed *cna-lacZ* gene products (COLN-BGAL) (Fig. 2) whose estimated *M_r* values were similar to those predicted from the length of *cna* present in the hybrid genes (Table 2). Only in one case were we unable to detect a COLN-BGAL polypeptide (Table 2).

As predicted from the results of plate tests (see above), β -galactosidase activity increased when cells harbouring the gene fusion plasmids were grown in the presence of mitomycin C (Table 2). Variations in basal and induced levels of β -galactosidase activity were observed both with *cna-lacZ* gene fusions and with *cna-lacZ* operon fusions obtained by the insertion of miniMu dII1681 or miniMu dII1728 into pAPIP401 (Table 2). There was no obvious correlation between fusion site and β -galactosidase activity other than the somewhat higher recovery of β -galactosidase with *lacZ* gene fusions in the extreme 5' end of *cna* (Table 2).

Regulation of *cna-lacZ* expression. The results of a detailed analysis of the SOS control of *cna-lacZ* expression confirmed that non-induced and mitomycin C-induced *cna-lacZ* expression are both dependent on the products of the *lexA* and *recA* genes (see Pugsley, 1984a), and are independent of the *recB*, *recF*, *uvrA* and *uvrB* gene products (data not shown). This is entirely consistent with the control of *cna* expression at the transcription level via the classical SOS regulatory circuit (reviewed by Walker, 1984).

Subcellular location of the *cna-lacZ* gene products. The insertion of miniMu d transposons into *cna* exerted a polar effect on the expression of the down-stream lysis gene, *cnl* (Pugsley, 1984a and Fig. 1). Cells carrying *cna-lacZ* fusion plasmids therefore did not lyse or release proteins when induced with mitomycin C. Less than 5% of the β -galactosidase activity was recovered

Table 2. *Characterization of cna-lacZ gene and operon fusions in derivatives of strains PAP488 (wild-type) or PAP489 (recA) carrying pAPIP401 cna::miniMu d plasmids*

Allele no.	Fusion site* (bp)	Fusion protein† (10 ⁻³ × M _r)	Colicin in fusion protein† (10 ⁻³ × M _r)	β-Galactosidase (Miller U)‡		
				Non-induced wild-type	Non-induced RecA	Induced wild-type
<i>Gene fusions</i>						
4	140	121	1	886	96	10600
2	250	123	3	837	25	25100
9	340	ND	ND	303	115	1800
5	340	124	4	184	44	5520
18	460	129	9	150	5	4420
15	480	137	17	123	6	1840
14	540	137	17	167	10	3170
3	620	147	27	128	33	4860
1	660	149	29	324	100	8740
19	880	154	34	208	9	4160
13	1000	156	36	201	20	5200
<i>Operon fusions</i>						
65	20			237	22	4000
62	240			544	82	13000
70	340			391	66	10900
66	640			544	82	11420
63	840			467	51	7900

ND, Not detected.

* The site of the miniMu d insertion in pAPIP401 was determined by restriction endonuclease digestion based on the known restriction maps of pAPIP401 and the miniMu d transposons (Fig. 1 and Castilho *et al.*, 1984). The length of the *cna* gene was estimated to be 1080 bp (Pugsley, 1984*a*); the fusion sites are represented relative to the estimated translation start point of *cna*.

† The size of the fusion protein was estimated by SDS-PAGE. The length of colicin N polypeptide was determined by subtracting 116000 (β-galactosidase) + 4000 (the peptide encoded by the 117 bp of Mu DNA in each fusion gene; Castilho *et al.*, 1984). Colicin N is 39000.

‡ β-Galactosidase activities were determined in cultures incubated to OD₆₀₀ 0.2 before the addition of 0.5 μg mitomycin C ml⁻¹, and then incubated for a further three generations. Results are means of three assays done with the same culture.

from the culture medium, and about 3% and 10% respectively of the cell-associated activity was recovered from the membrane fraction or was released by cold osmotic shock. These results are in agreement with others showing that colicins accumulate in the cytoplasm of producing cells (Cavard *et al.*, 1984).

Previous experiments indicated that pColE2-P9 could complement *cnl::Tn5* mutations in derivatives of pAPIP401, leading to the efficient release of colicin N and colicin E2 [unpublished data, see Pugsley & Schwartz (1983) for similar experiments with other Col plasmids]. We therefore transformed strains carrying pAPIP401 *cna::miniMu dII* plasmids with pColE2-P9 and studied the location of the COLN-BGAL hybrids and colicin E2 during growth in the presence of mitomycin C. The cells partially lysed and released colicin E2 within 2.5–3 h after the addition of mitomycin C to the culture, as is normal for cells carrying pColE2-P9 (Pugsley & Schwartz, 1984), but ≥93% of the β-galactosidase activity remained cell-associated. Less than 5% of this cell-associated activity was recovered from the membrane fraction of sonically disrupted cells. The distribution of the COLN-BGAL polypeptide, as detected by SDS-PAGE, was similar to that of the β-galactosidase activity (data not shown). We concluded that COLN-BGAL hybrids were cytoplasmic, and that they could not be released from producing cells through the colicin efflux pathway.

Fig. 1. Physical maps of plasmids used for constructing *cna-lacZ* and *cna-phoA* gene fusions. The maps of pColN-284 and pAPIP401 are based on those published previously (Pugsley, 1984a), with additional sites for *Bst*EII, *Eco*RV and *Kpn*I restriction endonucleases. The boxed area represents the region of pColN-284 which was subcloned into pBR322 to give pAPIP401 (Pugsley, 1984a). The physical map of pCHAP4 was constructed after analysis of fragments generated by digestion with various restriction endonucleases. The enzymes *Bgl*I, *Sac*I, *Eco*RI, *Bgl*II and *Sac*I did not cleave pCHAP4 DNA. Plasmid pCHAP12 was constructed by subcloning the smaller *Pst*I-*Bam*HI fragment of pCHAP4 (boxed region) into pUC9 cleaved with the same enzymes. The *cna-cni* operon in pCHAP12 is in the same orientation as the *lacZ* promoter. The location of the *cna* (colicin N), *cni* (immunity) and *cni* (lysis) genes in the boxed regions is shown at the top right of the figure (Pugsley, 1984a). The physical map of TnphoA is based on that of Manoil & Beckwith (1985), and is shown in the orientation in which it was found in pCHAP12::TnphoA plasmids.

Colicin N-alkaline phosphatase fusion proteins

Fusions between the *cna* and *phoA* genes. The transposon TnphoA carries a '*phoA* gene lacking sequences that code for the signal peptide. The insertion of TnphoA into the coding sequence of a gene can therefore create an in-frame fusion leading to the production of alkaline phosphatase. Export signals in the product of the target gene may induce the export of the hybrid protein (Manoil & Beckwith, 1985). *cna-phoA* gene fusions were obtained by TnphoA mutagenesis of pCHAP12, in which *cna* should be transcribed from both *lacZ* and *cna* promoters (Fig. 1). Twelve independent Col⁻ PhoA⁺ clones yielded four different classes of *cna-phoA* fusion plasmids (Table 3). The initial plasmids were stabilized by deleting IS50R (Methods), and then transferred to strain PAP488. The resulting strains all produced relatively high amounts of alkaline phosphatase activity [yields were similar to those obtained with strains carrying pCH2 (Hoffman & Wright, 1985)], and three of them produced large amounts of a novel polypeptide (COLN-PHOA) whose apparent *M_r* was in good agreement with that predicted for the corresponding *cna-phoA* gene product (Fig. 3, Table 3). Mitomycin C induction resulted in only

Fig. 2. Polypeptides encoded by selected *cna-lacZ* gene fusions. Cultures of strains carrying pAPIP401::miniMu dII plasmids were grown for three generations in L broth containing $0.5 \mu\text{g}$ mitomycin C ml^{-1} . The cells were then lysed by sonication in gel sample buffer and run on an SDS-PAGE gel. Only that part of the gel displaying the hybrid proteins is shown. The numbers at the top are the allele numbers of the respective *cna-lacZ* gene fusions. The M_r markers were β -galactosidase (116000) and myosin (205000).

Table 3. Characteristics of *cna-phoA* gene fusions in derivatives of strain PAP488 or PAP488(pMM100) carrying pCHAP12::TnphoA

<i>cna-phoA</i> allele no.	No. isolated	Fusion site* (bp)	Fusion protein† ($10^{-3} \times M_r$)	Colicin in in fusion protein† ($10^{-3} \times M_r$)	Alkaline phosphatase‡ (U per OD ₆₀₀ unit)			
					PAP488 non-induced	PAP488(pMM100) non-induced	PAP488(pMM100) + mitomycin C	PAP488(pMM100) + IPTG
101	8	200	ND	ND	160	6	190	6
104	1	250	56	9	21	5	40	14
112	2	500	64	17	446	8	504	360
105	1	800	76	29	219	6	276	205

ND, not detected.

* Positions of the TnphoA inserts are relative to the estimated translation start point of the *cna* gene (Pugsley, 1984a).

† The M_r of the hybrid protein was calculated from its migration in SDS-PAGE relative to proteins of known M_r . The amount of colicin N in the hybrid was calculated by deducting the M_r of alkaline phosphatase (47000) from that of the hybrid. Colicin N is 39000.

‡ Alkaline phosphatase activities were calculated in the same way as those of β -galactosidase (Miller, 1972). Non-induced cultures were at steady state during exponential growth. Induced cultures were growing in the presence of $0.5 \mu\text{g}$ mitomycin C ml^{-1} or 1 mM-isopropyl β -D-galactoside. PAP488(pCH2) produced 300–350 U alkaline phosphatase under the same conditions. Strain PAP488 itself produced <1 U alkaline phosphatase. The results are means of three assays done with the same culture. Activities varied in different experiments by as much as 30%.

Fig. 3. Total cell proteins of PAP488(pCHAP12::TnpHoA) derivatives grown in L broth. Cells were lysed by sonication in gel sample buffer and the proteins separated by SDS-PAGE. The samples are equivalent to 150 μ l of culture at OD₆₀₀ 1.0. A, Φ (cna-phoA)104; B, Φ (cna-phoA)105; C, Φ (cna-phoA)112; D, Φ (cna-phoA)101. Arrows indicate the putative cna-phoA gene products. The M_r marker proteins were bovine serum albumin (66000), phosphorylase *a* (97000) and ovalbumin (45000).

Table 4. Localization of alkaline phosphatase activity in PAP488 derivatives carrying pCHAP12::TnpHoA plasmids with or without pColE2-P9

Allele no.	Colicin in fusion protein ($10^{-3} \times M_r$)	Alkaline phosphatase activity (percentage of total)*							
		PAP488†					PAP488-(pColE2-P9)‡		
		Extracellular	Osmotic shock		Sonicated		Extracellular	Sonicated	
			Shock fluid	Cellular	Total soluble	Particulate		Soluble	Particulate
101	6§	6	43	51	46	54	70	22	8
104	9	3	18	79	34	66	22	48	30
112	17	4	13	79	29	71	43	49	8
105	29	6	32	62	45	55	45	41	14

* See Table 3 for specific activities; results are from a single experiment.

† In cultures grown to OD₆₀₀ 1.0 without induction.

‡ In cultures grown for 5 h in L broth containing mitomycin C, 80–90% of the colicin E2 activity was extracellular at this time.

§ Estimated from position of TnpHoA in cna gene. COLN-PHOA polypeptide was not detected in strains carrying this allele (see Table 3).

Fig. 4. Growth and protein release by strain PAP488(pColE2-P9 pCHAP12::Tn*phoA112*) growing in L broth containing 0.4% maltose to induce synthesis of amyloamaltase. Enzyme activities (○, amyloamaltase; ■, alkaline phosphatase; □, colicin E2) are the amounts which were cell-associated expressed as a percentage of the total activity present in the culture. The culture was induced with mitomycin C (0.5 µg ml⁻¹) at 0 min, and changes in culture density (OD₆₀₀, ●) were recorded. Results are from a single experiment.

a slight increase in *cna-phoA* expression. The introduction of pMM100 (*lacI*^q) into these strains reduced *cna-phoA* expression to basal levels, and restored inducibility by mitomycin C and by isopropyl β-D-galactoside (Table 3 and data not shown). These results showed that both *cna* and *lacZ* promoters could be active in the pCHAP12::Tn*phoA* derivatives.

The subcellular location of alkaline phosphatase activity and of COLN-PHOA polypeptide was determined by subjecting cells to osmotic shock to release periplasmic proteins, or by sonicating them and then centrifuging at high speed to separate soluble and particulate (membrane) fractions. Between 29% and 46% of the activity was detected in the soluble (periplasmic + cytoplasmic) fraction of the sonically-disrupted cells, and 13–43% of the activity was released by cold osmotic shock (Table 4). Wild-type alkaline phosphatase (strain PAP151) and hybrid β-lactamase-alkaline phosphatase encoded by the *bla-phoA* gene fusion in pCH2 (Hoffman & Wright, 1985) were almost exclusively soluble and were released by cold osmotic shock. In contrast, the analysis of the protein content of the various cell fractions by SDS-PAGE indicated that COLN-PHOA was located exclusively in the particulate fraction (data not shown). Identical results were obtained when strain PAP308 [*ompF*; lacking the colicin N receptor protein (Tommasen *et al.*, 1984)] was used as host for the *cna-phoA* fusion plasmids. These results ruled out possible artifacts caused by the readsorption of released COLN-PHOA by the colicin N receptor. Possible explanations for the different locations of active alkaline phosphatase and COLN-PHOA polypeptide are given in the Discussion.

Strains carrying *cna-phoA* fusion plasmids together with pColE2-P9 partially lysed after 2–2.5 h growth in the presence of mitomycin C. Alkaline phosphatase activity was also detected in the growth medium, although it was released more slowly and less extensively than colicin E2 (Fig. 4 and Table 4). Furthermore, when the protein content of the induced cells and culture medium was examined, the COLN-PHOA polypeptide was found exclusively in the cells, whereas the bulk of the colicin E2 was in the medium (data not shown). Amyloamaltase, a maltose-inducible cytoplasmic protein remained wholly cell-associated throughout the period during which colicin was being released from the cells (Fig. 4).

DISCUSSION

The data we present here indicate that hybrid proteins containing the NH₂-terminal part of colicin N and almost the entire sequence of alkaline phosphatase or β -galactosidase can be made in large amounts by *E. coli* K12 carrying the appropriate fused genes. To our knowledge, this is the first time that such large quantities of colicin fusion proteins have been obtained, although Vernet *et al.* (1985) have described a colicin E3–insulin hybrid which is synthesized in relatively low amounts, and Yamade & Nakazawa (1984) have described fusions between the *ceaA* (colicin E1) and *lacZ* genes.

None of the COLN–BGAL or COLN–PHOA chimeras were secreted into the growth medium by cells that were actively releasing colicin E2. These results will now be considered in the light of what is already known about the proteins which comprise the hybrids, and of previous attempts to export other β -galactosidase or alkaline phosphatase hybrids.

The system involved in colicin efflux is completely different from the signal peptide-dependent export of periplasmic and outer membrane proteins. Therefore, even though β -galactosidase apparently cannot be made wholly extracytoplasmic by genetically fusing it to proteins which are made with signal sequences [Pugsley & Schwartz, 1985; see also Benson *et al.* (1985) and Tommassen *et al.* (1985) for further discussion of this point], there was no *a priori* reason to expect that the same would be true for COLN–BGAL hybrids. However, COLN–BGAL hybrids containing almost all of the colicin N polypeptide were fully retained within the cytoplasm of cells that were releasing colicin.

In choosing the normally periplasmic alkaline phosphatase as an alternative to β -galactosidase for fusing to colicin N, we hoped to avoid problems caused by the possible inability of the LacZ sequences to cross bacterial membranes. Alkaline phosphatase is enzymically inactive when it remains in the cytoplasm, presumably because the reducing conditions in the cytoplasm prevent the formation of disulphide bridges required to produce active alkaline phosphatase (Michaelis *et al.*, 1983; Hoffman & Wright, 1985; Manoil & Beckwith, 1985). We therefore anticipated that COLN–PHOA hybrids would have only low alkaline phosphatase activities because they, like the colicin itself, would accumulate at least transiently in the cytoplasm. To facilitate their detection we used a constitutively expressed *cna* gene as the target for *TnphoA* mutagenesis. This approach was successful in that we obtained *cna-phoA* gene fusions which encoded enzymically-active COLN–PHOA, but the hybrid proteins exhibited several unusual characteristics. The most important of these was that, in three of the four cases studied, the COLN–PHOA polypeptide was detected only in the particulate cell fraction, whereas a substantial amount of the active enzyme was detected in the soluble cell fraction. Thus, the specific activity of the COLN–PHOA hybrids varied according to their location within the producing cells. We suspect that these COLN–PHOA polypeptides were mostly in an aggregated state rather than in the membrane, as is the case with some other over-produced proteins in *E. coli* (Schoemaker *et al.*, 1985). Active alkaline phosphatase was also present in the soluble cell fraction of cells carrying the (*cna-phoA*)101 gene fusion, although no COLN–PHOA polypeptide could be detected, presumably because it was proteolytically degraded. Both of these observations could explain why COLN–PHOA polypeptides were not released into the medium even though relatively high levels of alkaline phosphatase activity were detected. Some of these anomalies could have arisen as a consequence of the high level expression of the *cna-phoA* fusions, or because we only studied potential *cna-phoA* fusions which encoded active alkaline phosphatase.

An alternative interpretation of our data is that the amino-terminal 70% colicin N does not contain all of the signals required for hybrid protein release into the medium. Further studies with different types of colicin gene fusions and with mutationally-altered colicins specifically affected in colicin efflux will be needed to identify sequences involved in the release of colicins from producing cells.

Colicins are translocated into as well as out of *E. coli* cells. The two processes are apparently completely different, although there are some obvious similarities between the mechanisms of colicin uptake and the uptake of nuclear-encoded proteins by mitochondria (Pugsley, 1984b; Hay *et al.*, 1984). Studies of mitochondrial biogenesis have been considerably advanced through

the use of hybrid proteins (Douglas *et al.*, 1984; Hurt *et al.*, 1984). The COLN-BGAL hybrids that we describe here may be useful for studying the mechanisms of colicin uptake, particularly since colicin N, unlike most other colicins, uses an abundant outer membrane protein as its receptor (Tommassen *et al.*, 1984), thereby increasing the number of colicin molecules that could penetrate into the cell.

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