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Short communication

Heterologous ΦX174 gene E-expression in *Ralstonia eutropha*: E-mediated lysis is not restricted to γ-subclass of *proteobacteria*

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Abstract

E-lysis of *Ralstonia eutropha* H16, which belongs to the β-subclass, was undertaken to verify whether transmembrane tunnel formation is possible in bacteria which do not belong to the enterobacteriaceae. For this purpose, a new gene E expression plasmid, pKG12, with two origins of replication, *oriV* and *oriT*, from plasmid pRP4, chloramphenicol and kanamycin resistance genes and a casette composed of *λcI857* and *λpR* gene *E* was constructed. Temperature upshift of *R. eutropha* H16 (pKG12) from 28 to 45°C during exponential growth resulted in lysis of the strain with features characteristic of E-mediated lysis of *Escherichia coli*. The cytoplasmic contents released can easily be separated from the still intact envelope fraction by centrifugation or filtration. As *R. eutropha* H16 represents an important industrial organism, E-mediated lysis could facilitate procedures for the recovery of intracellular mediators or products like polyhydroxyalkanoates.

Keywords: Bacteriophage ΦX174 lysis gene E; *Ralstonia eutropha* H16; Cell lysis; Heterologous gene expression; Polyhydroxyalkanoates

1. Introduction

In nature, the single stranded DNA phage ΦX174 has a very restricted host range and infects only *Escherichia coli* or *Salmonella typhimurium*. Subcloning the ΦX174 genome has shown that

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expression of gene E of the phage is sufficient to lyse *E. coli* by formation of a trans-membrane tunnel structure (Witte et al., 1990). In Gram-positive organisms, the E-mediated lysis mechanism is not active as E-cell lysis is coupled to a fusion process of inner and outer membranes. However, in this group of bacteria, protein E kills cells (Halfmann et al., 1993).

So far, heterologous expression of cloned gene E in bacteria not related to the host range of phage ΦX174 has been successful in several enterobacteriaceae: *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Actinobacillus pleuropneumoniae* (Szostak et al., 1995). Recently, E-mediated lysis from plasmids with *E. coli* related origins has been achieved in *Bordetella bronchiseptica*, which, like *R. eutropha*, is also a member of the β-subclass of *Proteobacteria* (Klein, R., Lubitz, W., unpublished results). Investigations of the molecular mechanism of protein E-mediated lysis of *E. coli* indicated a specific lysis mechanism characterized by formation of a transmembrane tunnel structure with fused inner and outer membranes at its borders (Witte et al., 1990; Schön et al., 1995). The resulting bacterial ghosts, consisting of intact structures from the cytoplasmic membrane to the surface of the cell envelope, retain their rigid structure and can be removed from the supernatant by filtration or centrifugation (Witte et al., 1991).

Ralstonia eutropha H16 is a well characterized bacterial strain used for industrial production of polyhydroxyalkanoates (PHAs), e.g. copolymers of polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHV) (Byrom, 1990). The *R. eutropha* operon responsible for PHB/PHV formation has been cloned and sequenced (Schubert et al., 1988, 1991; Slater et al., 1988; Peoples and Sinskey, 1989). Heterologous expression of genetically engineered PHB genes in *E. coli* leads to very efficient production of PHB (Kalousek et al., 1993; Kalousek and Lubitz, 1995). The heterologous gene expression of the PHB operon indicates that transcriptional and translational signals of *R. eutropha*, a member of the β-subclass of *Proteobacteria* (Busse and Auling, 1992), are also recognized by enterobacteria which belong to the γ-subclass (Brenner, 1992). In addition, gene transfer in *R. eutropha* has been achieved by mobilization of derivatives of the broad host range plasmid pRP4 (Hrabak, 1987).

E-mediated release of PHA in *E. coli* (Resch et al., 1998) stimulated the present investigations of the application of the E-lysis system to a natural PHA producer strain. It was shown that E-mediated lysis of *R. eutropha* is very similar to that of *E. coli*. Considering the success of inducing PHB formation at the exponential growth phase in recombinant *E. coli* (Kalousek and Lubitz, 1995) it seems feasible that E-lysis of *R. eutropha* can be applied for aqueous PHB release in genetically modified *R. eutropha* strains. In this communication the question is raised whether the E-specific lysis system of the *E. coli*-phage ΦX174 can be applied to *R. eutropha* strain H16. The advantage of the E-specific lysis mechanism for PHA release in *E. coli* is that endotoxin (lipopolysaccharide from the outer membrane) can be easily removed from the polymer and that PHA can be recovered in its natural semifluid conformation in an aqueous environment (Lubitz, 1992). This aqueous purification procedure contrasts with the common chemical methods used for PHA extraction from producer strains (Doi, 1990).

2. Materials and methods

2.1. Bacteria and plasmids

R. eutropha H16 (DSM #428) was obtained from the German Collection of Microorganisms (DSM). Plasmid pSH2 (Resch et al., 1998) pWIK100 (Krek, 1986) and pRK2013 (Ditta et al., 1980) have been described. Plasmid pKG12 was constructed by inserting a 4.5 kb *Pst*I fragment of pWIK100 carrying *oriT* (transfer function) and *oriV* from the broad host range vector pRP4 and the chloramphenicol resistance gene (Hrabak, 1987) into the *Pst*I site downstream *plac* of plasmid pSH2 (Fig. 1).

2.2. DNA manipulations

DNA manipulations were done according to methods described by Maniatis et al. (1982) or by

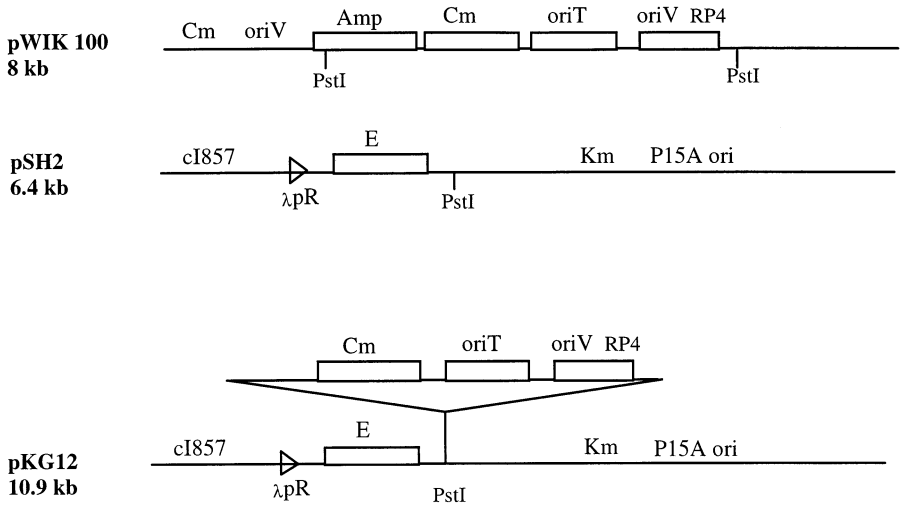

Fig. 1. Construction of pKG 12. The 4.5 kb *Pst*I fragment of pWIK100 containing *oriV* and *oriT* from the broad host range vector RP4, accepted by *R. eutropha* H16, was ligated into the *Pst*I site of the E-lysis plasmid pSH2 resulting in the specific lysis plasmid pKG12. Expression of the lysis gene *E* is regulated by the λ-promotor *λpR* under control of the thermosensitive *cI857* λ-repressor. Both selection marker genes for resistance to chloramphenicol and kanamycin were contained on the final construct pKG12.

following the recommendations of enzyme manufacturers.

2.3. Growth conditions

R. eutropha H16 (pKG 12) was grown in Luria broth (LB) consisting of 10 g l^{-1} peptone from caseine, 5 g l^{-1} yeast extract and 10 g l^{-1} NaCl containing 75 μg ml^{-1} kanamycin and 75 μg ml^{-1} chloramphenicol at 28°C. *R. eutropha* H16 (pWIK100) was grown in LB with 75 μg ml^{-1} chloramphenicol and 150 μg ml^{-1} ampicillin. The strains were grown in shake flasks equipped with a cuvette containing 30 ml of media aerated by a magnetic stirrer at 500 rpm.

2.4. Transfer of plasmids into R. eutropha H16

For transfer of plasmids into *R. eutropha* H16 the $CaCl_2$ method (Maniatis et al., 1982), triparental mating (Ditta et al., 1980) and electroporation (Bio Rad Gene Pulser; capacitance at 25 μF and Pulse Controller at 200 Ω) according to Wirth et al. (1989) were used. After incubation of the transformation mixture for 1 h in antibiotic-free LB medium under gentle shaking at 28°C, cells were transferred to LB-plates containing 75 μg ml^{-1} kanamycin and 75 μg ml^{-1} chloramphenicol and incubated at 28°C.

2.5. High resolution scanning electron microscopy

Scanning electron micrographs were taken with a Hitachi S-800 field emission scanning microscope (Hitachi, Tokyo, Japan). Fixation of cells and preparation for electron microscopy was done as described in Witte et al. (1990).

3. Results and discussion

For E-mediated lysis of *E. coli* a variety of plasmids can be used, which are characterized by expression of gene E from an inducible promotor using either the *plac* or *λ pL/pR* promoters and corresponding *lac* i^q or *cI857* repressor, respectively (Henrich et al., 1982; Bläsi et al., 1990; Szostak et al., 1995). First attempts to transform *R. eutropha* H16 with plasmid pSH2 (Fig. 1) by the conventional $CaCl_2$ method or electroporation were not successful. As direct transformation of *R. eutropha* H16 with pKG12 by the conventional

$CaCl_2$ method failed, plasmid pKG12 was transformed into *E. coli* HB101 and transferred via triparental mating (Ditta et al., 1980) into *R. eutropha*. For this purpose, *E. coli* HB101 (pKG12) was cotransformed with the mobilization helper plasmid pRK2013. Conjugational transfer of pKG12 from *E. coli* (pKG12, pRK2013) into *R. eutropha* H16 occurred at a very low frequency. Alternatively, transformation of *R. eutropha* H16 with pKG12 was achieved by electroporation giving rise to approximately 100 transformants per μg of pKG12 DNA.

Although the transformation efficiency with both methods was poor, lysis tests of different *R. eutropha* H16 (pKG12) clones showed that all clones lysed with an efficiency more than 95% of the cells. Instead of a temperature upshift to 42°C which is normally used for *E. coli*, a shift of the culture samples from 28 to 45°C had to be used (Fig. 2). The growth rate of *R. eutropha* H16 (pWIK100) was approximately half of that for H16 (pKG12) at 28 and 45°C. Lysis onset of *R. eutropha* H16 (pKG12) occurred approximately 15 min after temperature upshift from 28 to 45°C, and progressed with a lysis rate (Leduc and van Heijenoorth, 1980) of k_L 1×10^{-3} min^{-1}, comparable to that of the growth rate. Onset of E-mediated lysis in *E. coli* depends on the growth rate of the bacteria (Lubitz et al., 1984) and starts under optimal growth conditions approximately 10 min after temperature upshift (Witte et al., 1993). Lysis of *R. eutropha* H16 (pKG12) is specific for the onset of gene E expression and not a result of the raised incubation temperature, as the control *R. eutropha* H16 (pWIK100) increased in optical density at the higher temperature (Fig. 2).

Growth of *R. eutropha* H16 is optimal at 30°C (Kersters and De Ley, 1983) very close to the 28°C used for growth of *R. eutropha* H16 (pKG12), under conditions where gene E expression is repressed by the *cI857* repressor molecules at the λpR operator site (Villaverde et al., 1993). The growth rates of *R. eutropha* H16 transformed with pKG12, however, were very close to the growth rates observed for untransformed cells. Electron microscopic investigations of *R. eutropha* H16 (pKG12) grown at 28°C (Fig. 3A) and of E-lysed samples taken approximately 150 min after temperature upshift (Fig. 3B) revealed that the cell length of *R. eutropha* under these conditions increased 2 to 3 times over normal size. As in *E. coli* (Witte et al., 1990, 1992) E-lysis holes seen in

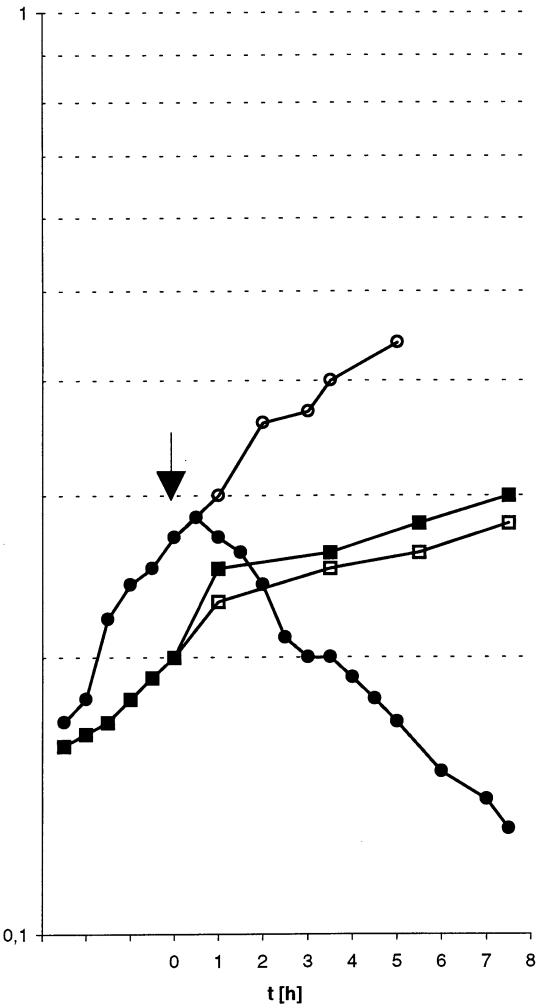

Fig. 2. Lysis graph of *R. eutropha* H16 transformed with pKG12. E-mediated lysis of *R. eutropha* H16 (pKG12) was induced by temperature shift from 28 to 45°C as indicated by the arrow (↓). *R. eutropha* H16 (pKG12) grown at 28°C (○) showed normal growth behaviour. Approximately 15 min after the temperature shift, onset of lysis with a lysis rate, indicated by decrease of the optical density, comparable to the growth rate occurred (●). Probes were kept at 45°C during the lysis process. Control strain *R. eutropha* H16 (pWIK100) grown at 28°C (□) and 45°C (■) respectively, exhibited lower growth rates in comparison to the lysis strain. The difference between growth rates at 28 and 45°C in the control was not significant.

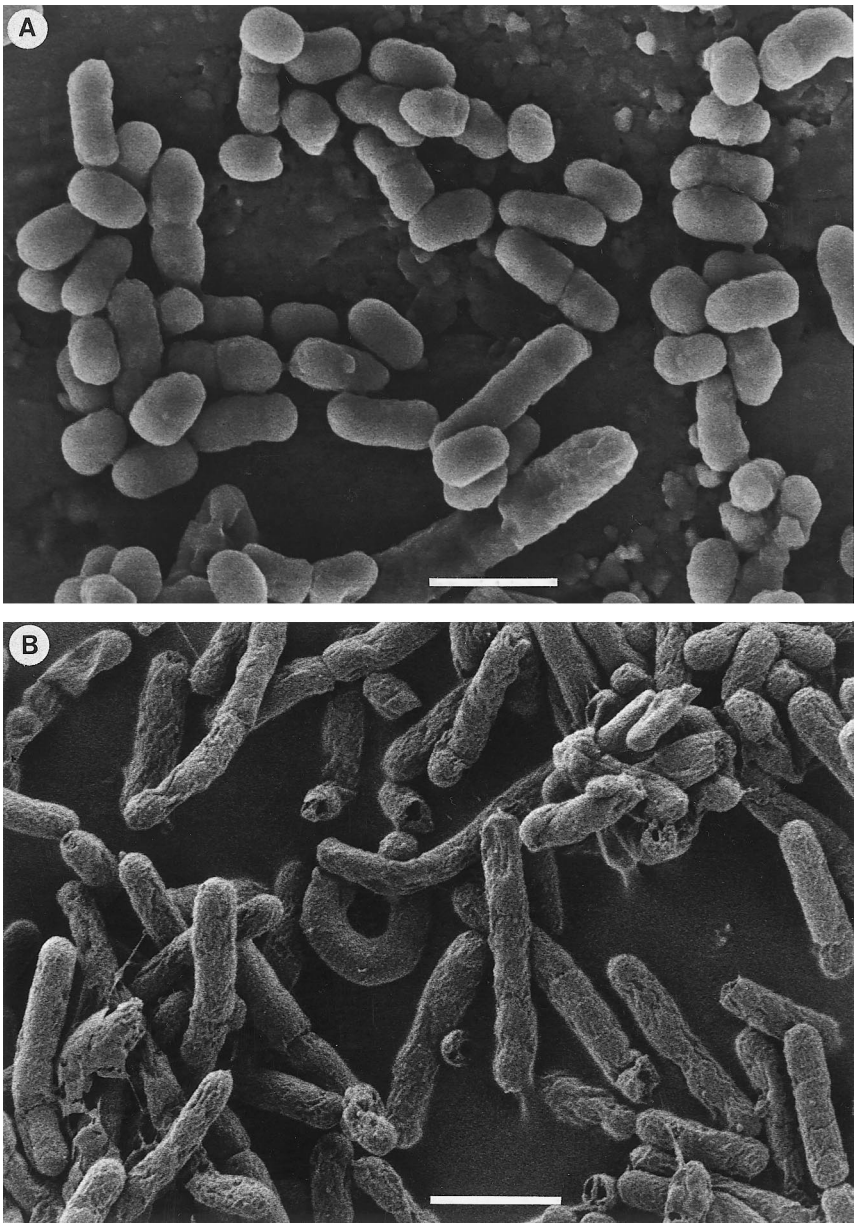

Fig. 3. A. High resolution field emission scanning electron micrograph of *R. eutropha* H16 (pKG12) control without thermal induction. B. *R. eutropha* H16 (pKG12) cells after decrease of optical density at the end of the lysis procedure, showing lysis tunnel structures at the cell pole region. Also a pole cap bursted off from a cell can be seen above the space bar. The white space bar represents 2 μm.

R. eutropha ghosts are located in areas of potential cell division, such as the middle septum or at polar sites of the cells (Fig. 3B). It is worth noting that E-mediated lysis of *R. eutropha* did not change the rod-like morphology of the ghosts although cell elongation occurred at the raised growth temperature (Fig. 3A). E-mediated lysis of Gram-negative bacteria other than *E. coli* does

not always maintain the morphology of the bacterial ghosts. In *V. cholerae*, the usually comma-shaped bacteria 'round up' prior to lysis (Eko et al., 1994). Electron microscopic inspection of *R. eutropha* ghosts from different experiments indicate that more than 95% of the total *R. eutropha* cell population is lysed by the E-mediated mechanism. E-specific lysis holes visible in Ralstonia ghosts vary in diameter from 150 to 300 nm. For *E. coli* ghosts a variation of 40 and 200 nm is reported (Witte et al., 1992).

The results presented here indicate that plasmid borne E-mediated lysis is not only restricted to natural hosts of ΦX174 or the enterobacteriaceae (β-subclass) but may likely be used for most common members of biotechnologically used Gram-negative bacteria. Thus, it represents a widely applicable method for cell disruption and may be used alternatively to sonication, enzymatic digestion, or French press procedures. The release of natural or recombinant intracellular proteins from *E. coli* by E-mediated lysis can be achieved with high yield as exemplified for β-galactosidase (Witte and Lubitz, 1989) or human prourokinase (Bläsi et al., 1990). The advantage of this release system is that the bacterial envelope remains intact and can easily be removed from the supernatant.

One application of E-mediated lysis of *R. eutropha* with biotechnological implication is the release of PHA-polymers. There is a large body of knowledge concerning fermentation and optimization of PHA production with *R. eutropha* during the stationary growth phase. However, as in *E. coli*, stationary growth of *R. eutropha* H16 does not allow protein E-mediated lysis, as this process is dependent on cellular factors involved in cell division (Witte et al., 1993). Thus, E-mediated lysis may at present not be a suitable mechanism to release PHB from *R. eutropha* as the natural gene regulation of the PHB-operon somehow senses the growth phase of the cells and is not induced during exponential growth. However, by separating the PHB production genes from their natural promoter and exchanging the ribosome binding sites, a promising strategy has recently been worked out which allows PHB-production at a rather high rate during exponential growth in *E. coli* (Kalousek and Lubitz, 1995). Therefore, it seems feasible to engineer the regulatory circuits of PHB formation in *R. eutropha* by analogy to *E. coli*. The aim is to apply the alternative lysis method by inhibition of lysis with $MgSO_4$ (Lubitz, 1992). Although recombinant production of PHA by *E. coli* is very effective (Kalousek et al., 1993), *R. eutropha* still is the standard organism for PHB mass production. The results described in this communication encourage us to develop a simple aqueous purification and recovery system for PHA granules from its natural producer *Ralstonia eutropha*.

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