The expression of the *Acinetobacter calcoaceticus* *recA* gene increases in response to DNA damage independently of RecA and of development of competence for natural transformation

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Using the *lacZ* operon fusion technique, the transcriptional control of the *Acinetobacter calcoaceticus* *recA* gene was studied. A low (approximately twofold) inductive capacity was observed for compounds that damage DNA and/or inhibit DNA replication, e.g. methyl methanesulfonate, mitomycin C, UV light and nalidixic acid. Induction of the *recA* gene by DNA damage was independent of functional RecA. The presence of the *recA* promoter region on a multicopy plasmid had the same effect on *recA* transcription as the presence of DNA-damaging agents. Thus, *recA* expression in *A. calcoaceticus* appears to be regulated in a novel fashion, possibly involving a non-LexA-like repressor. Regulation of the *recA* gene in *A. calcoaceticus* appears not to be part of a regulon responsible for competence for natural transformation: in cells exhibiting extremely low transformation frequencies, the level of transcription of the *recA* gene was found to be comparable to the level found in cells in the state of maximal competence.

Keywords: *Acinetobacter calcoaceticus*, *recA* gene, DNA damage, natural transformation, competence

INTRODUCTION

RecA is the major enzyme involved in homologous recombination (Cox, 1991). The *recA* gene from approximately 35 different organisms has been cloned and completely or partially sequenced (for a review, see Miller & Kokjohn, 1990). Among these are nine naturally transformable organisms: the Gram-negative organisms *Acinetobacter calcoaceticus* (Gregg-Jolly & Ornston, 1994), *Azotobacter vinelandii* (Venkatesh & Das, 1992), *Campylobacter jejuni* (Guerry *et al.*, 1994), *Haemophilus influenzae* (Zulty & Barcak, 1993), *Neisseria gonorrhoeae* (Story *et al.*, 1993), *Pseudomonas stutzeri* (Vosman *et al.*, 1993) and

Thiobacillus ferrooxidans (Ramesar *et al.*, 1989) and the Gram-positive bacteria *Streptococcus pneumoniae* (Dybvig *et al.*, 1992; Martin *et al.*, 1992) and *Bacillus subtilis* (Stranathan *et al.*, 1990). In *Escherichia coli*, *recA* expression is induced by the presence of single-stranded DNA (ssDNA) resulting, for instance, from DNA damage (Higashitani *et al.*, 1992). In short, the RecA protein binds to ssDNA, forming a nucleoprotein filament (Story *et al.*, 1993). The filament enhances the autoprotease activity of LexA. Cleaved LexA subsequently derepresses a large number of genes involved in repair of DNA damage, the so-called SOS response genes, including *recA* and *lexA* (Little & Mount, 1982).

In natural transformation, incoming homologous chromosomal DNA has to become incorporated into the recipient's chromosome before genetic determinants, present on this DNA, can be expressed. Therefore, natural transformation is also dependent on homologous recombination. The importance of RecA in natural transformation is underlined by the observation that expression of the *recA* gene of *B. subtilis*, *S. pneumoniae* and

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Abbreviations: MMS, methyl methanesulfonate; MU, Miller units; ssDNA, single-stranded DNA.

Streptococcus sanguis (all Gram-positive bacteria) is induced upon induction of competence (Wise *et al.*, 1973; De Vos & Venema, 1982; Raina & Macrina, 1982). The *recA* gene of both *B. subtilis* and *S. pneumoniae* is subject to dual regulation (Cheo *et al.*, 1991, 1993; Raymond-Denise & Guillen, 1992; Pearce *et al.*, 1995; Martin *et al.*, 1995). In these organisms, transcription of *recA* is regulated both by an SOS response [dependent on functional RecA (RecE) and LexA (DinR)] and by the regulatory pathway that controls competence development for natural transformation (independent of functional RecA).

With respect to the naturally transformable Gram-negative bacteria, much less is known about the regulation of *recA* transcription in relation to induction of competence. This paper describes an analysis of *A. calcoaceticus* *recA* transcriptional regulation using the *lacZ* operon fusion technique. Induction of *recA* transcription was studied in particular in response to (i) DNA damage, (ii) development of competence for natural transformation and (iii) the presence of functional RecA.

METHODS

Bacteria, plasmids and growth conditions. Strains and plasmids used in this study are listed in Table 1. Plasmid pAKA1024-32 is based on the *E. coli* vector pUN121 (Nilsson *et al.*, 1983) and contains a 3.5 kb *EcoRI* fragment with the chloramphenicol-resistance gene from pKT210 (Bagdasarian *et al.*, 1981), flanked by two 2.1 kb regions from the *A. calcoaceticus* BD413 chromosome. In this construct, the 3.5 kb *EcoRI* fragment replaces a 4.3 kb region of the *A. calcoaceticus* BD413 chromosome, containing the *estA* gene (Kok *et al.*, 1993). The chloramphenicol-resistance gene is efficiently inserted into the *A. calcoaceticus* BD413 chromosome via homologous recombination in these flanking regions. Plasmid pARA0 is based on pUN121 and contains a chromosomal 2.4 kb *HindIII* fragment with the *A. calcoaceticus* BD413 *recA* gene. Plasmid pAVP21

contains the promoterless *E. coli lacZ* gene from pJO220, followed by the *nptII* gene (encoding kanamycin resistance) from pUC4K (Vieira & Messing, 1982). Plasmid pWH1274 is an *E. coli*-*A. calcoaceticus* shuttle vector (Hunger *et al.*, 1990).

E. coli strains were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). *A. calcoaceticus* was grown in batch cultures in LB medium or in minimal medium at 30 °C with aeration in a rotary shaker. *Acinetobacter* minimal medium and minimal agar were prepared according to Juni (1974). They contained 60 mM lactic acid, 11 mM KH_2PO_4 , 95 mM Na_2HPO_4 , 0.81 mM MgSO_4 , 37 mM NH_4Cl , 0.068 mM CaCl_2 , 1.8 μM FeSO_4 and 1.5% (w/v) agar for the latter. The *A. calcoaceticus* chemostat medium consisted of minimal medium (Juni, 1974) with Vishniac trace element solution (1 ml l⁻¹) and 15 μg kanamycin ml⁻¹. One litre of Vishniac trace element solution contained: 1 g Na-EDTA, 2.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 50 mg H_3BO_3 , 150 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 150 mg $\text{CuCl}_2 \cdot 5\text{H}_2\text{O}$, 25 mg $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ and 50 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. The chemostat experiments were performed in a Bioflo model C30 fermenter (New Brunswick Scientific) at 30 °C. The pH was regulated at 7.0 by titration with 1 M HCl. The working volume of the culture vessel was 340 ml. The cultures were aerated with a flow of 1.2 l air min⁻¹, with a stirring rate of 400 r.p.m.

When appropriate, media were supplemented with antibiotics in the following amounts (unless stated otherwise): ampicillin, 100 μg ml⁻¹ (*E. coli*) or 200 μg ml⁻¹ (*A. calcoaceticus*); chloramphenicol, 50 μg ml⁻¹ (*A. calcoaceticus*); kanamycin, 50 μg ml⁻¹ (*E. coli*) or 15 μg ml⁻¹ (*A. calcoaceticus*).

***A. calcoaceticus* transformations.** *A. calcoaceticus* strains were grown to competence as follows: an overnight culture in LB medium was diluted 1:25 in fresh LB medium and cultured for an additional 2 h at 30 °C. At this stage, the culture is competent for genetic transformation. When transformation frequencies of cultures were to be determined (as a measure of the level of competence for natural transformation), the transformation protocol used was as follows. A culture (0.5 ml) was incubated for 60 min with 2 μg pAKA1024-32. Subsequently, 50 μg DNase I was added to terminate further DNA uptake and the transformation mixture was incubated for an additional 90 min to allow expression of the transformed chloramphenicol-

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant geno/phenotype*	Reference
Strains		
<i>A. calcoaceticus</i> BD413	Wild-type	Juni (1972)
<i>A. calcoaceticus</i> AAC406	<i>recA::lacZ::nptII</i>	This study
<i>A. calcoaceticus</i> AAC407	<i>recA⁺ recA::lacZ::nptII</i>	This study
<i>E. coli</i> DH5 α	<i>recA1</i>	Hanahan (1983)
<i>E. coli</i> AB1157	<i>recA⁺</i>	B. J. Bachmann, Yale University, CT, USA
Plasmids		
pAKA1024-32	Cm ^r	Kok <i>et al.</i> (1993)
pAVP21	<i>lacZ::nptII</i>	Vosman <i>et al.</i> (1993)
pARA0	Amp ^r <i>recA⁺</i>	Palmen <i>et al.</i> (1992)
pARA4	Amp ^r <i>recA::lacZ::nptII</i>	This study
pARA11	Amp ^r	This study
pWH1274	Amp ^r	Hunger <i>et al.</i> (1990)

* Amp^r and Cm^r refer to resistance to ampicillin and chloramphenicol, respectively.

Fig. 1. Restriction maps of the chromosomal *recA* region of the recombinant *A. calcoaceticus* strains AAC406 (a) and AAC407 (b) and of the chromosomal inserts in pARA4 and pARA11 (c). Ev, EcoRV; H, HindIII; S, SalI.

resistance marker. In all other transformations (carried out with several different plasmid-DNA samples), the incubation time with DNA was extended to 3 h and no DNase I was added. Transformants were selected on LB plates containing the appropriate antibiotic(s).

DNA techniques. Chromosomal DNA was isolated from *A. calcoaceticus* as described by Vosman & Hellingwerf (1991). Plasmid DNA was purified from *A. calcoaceticus* using lysis by boiling (Sambrook *et al.*, 1989). All additional molecular genetic techniques were carried out according to standard protocols (Sambrook *et al.*, 1989). In the cloning procedures, enzymes and reagents were used as recommended by the manufacturer (Pharmacia LKB Biotechnology AB).

Construction of the *recA::lacZ* fusion strains. The unique *SalI* restriction site in the *recA* gene (Palmen *et al.*, 1992; Gregg-Jolly & Ornston, 1994) in pARA0 was used to insert the *lacZ::nptII* cassette from pAVP21 as a 4.5 kb *SalI* fragment. This resulted in plasmid pARA4 (see also Fig. 1c). It has been shown that insertion of an *nptII* gene in this site in the chromosome of *A. calcoaceticus* leads to a RecA-deficient phenotype (Palmen *et al.*, 1992). As expected, *E. coli* DH5 α , containing pARA4, was sensitive to the addition of 0.03% methyl methanesulfonate (MMS) and resistant to kanamycin. Furthermore, this strain showed high-level expression of β -galactosidase, in contrast to *E. coli*(pARA3). The plasmid in this latter strain is identical to pARA4, except that it contains the 4.5 kb *SalI* fragment, bearing *lacZ::nptII*, in the opposite orientation. Restriction analyses, based on nucleotide sequence information (Gregg-Jolly & Ornston, 1994), confirmed that pARA4 has the *lacZ* gene under control of the *recA* promoter.

To construct chromosomal *recA::lacZ* fusion strains, pARA4 was introduced into *A. calcoaceticus* BD413 via natural transformation. Plasmids based on pUN121 do not replicate in *A. calcoaceticus*. Kanamycin-resistant, β -galactosidase-producing transformants can only be formed by integration of the *lacZ::nptII* cassette via homologous recombination, facilitated by the *recA* sequences, into the host chromosome. Basically, integration can occur via two recombination events: (i) via replacement recombination, resulting from homologous recombination on both flanking sequences of the *lacZ::nptII* cassette, or (ii) via a Campbell-like mode of insertion, resulting

from a single recombination event on one of the two flanking sequences. In the case of a Campbell-like integration, the entire plasmid will be integrated, whereas replacement recombination results in insertion of the *lacZ::nptII* cassette only and in disruption of *recA*. Replacement recombinants will therefore be kanamycin-resistant and RecA-deficient. After transformation of BD413 with pARA4, strains with a kanamycin-resistant phenotype were selected and screened for sensitivity towards MMS and ampicillin. Restriction analysis combined with Southern hybridization showed that the organization of the *recA* region was as expected (Fig. 1a). Two strains were initially selected for further use (AAC406/1 and AAC406/2).

Campbell-like integration (also termed duplication insertion) results in duplication of the homologous DNA fragment on the plasmid taking part in the insertional recombination reaction. Plasmid pARA4 contains two homologous fragments, of 1.5 and 0.9 kb, flanking the inserted *lacZ::nptII* cassette. Therefore, two types of transformants were expected. One type would result from recombination on the 1.5 kb homologous fragment and would possess the chromosomal gene arrangement depicted for AAC407 in Fig. 1(b). Recombination on the 0.9 kb homologous fragment of pARA4 would result in a reverse gene arrangement, i.e. the wild-type *recA* gene would precede the *recA::lacZ* fusion. Transformants having pARA4 integrated according to a Campbell-like mechanism will be kanamycin- and ampicillin-resistant, and RecA-proficient. After transformation of BD413 with pARA4, transformants resistant to MMS, kanamycin and ampicillin were selected. Restriction analysis combined with Southern hybridization of three of these transformants confirmed that in all three of them, the chromosomal *recA* region is as shown in Fig. 1(b). For this study, this is also the preferred arrangement because all possible regulatory signals upstream of the *recA::lacZ* promoter are conserved. Also, for the Campbell-like integration, two strains were initially used for further study (AAC407/1 and AAC407/2). However, both the AAC406 and the AAC407 set of strains did not produce significantly different data sets. Therefore, no distinction will be made between the two isogenic strains. Their data sets have been combined and the four strains will be referred to as AAC406 and AAC407, respectively, below.

Construction of the *recA* promoter plasmid. A plasmid that was able to replicate in *A. calcoaceticus* and that contained the

promoter region of the *A. calcoaceticus recA* gene, pARA11, was constructed by ligating the 1.5 kb *HindIII*–*SalI* fragment from pARA0 (Palmen *et al.*, 1992; Fig. 1c) into pWH1274 (Hunger *et al.*, 1990). The 1.5 kb fragment contains 0.4 kb of the 5'-end of the *recA* gene and 1.1 kb of upstream sequence.

Measurement of *recA::lacZ* transcription levels. β -Galactosidase levels were determined according to Miller (1982) and are expressed in Miller units (MU). Levels of β -galactosidase presented are always the mean of duplicate measurements.

The effects of different DNA-damaging agents on *recA* transcription were determined as follows. An overnight culture of a *recA::lacZ* operon fusion strain in LB medium, containing the appropriate antibiotic(s), was diluted 1:25 into fresh medium. After 2 h growth at 30 °C, a DNA-damaging agent was added to the culture, which was incubated for an additional 2 h before samples for the β -galactosidase activity measurements were taken. UV irradiation (254 nm) was carried out on 6 ml samples of a culture in open Petri dishes (9 cm diameter).

Protein concentrations were determined according to Bradford (1976) using Bradford reagent from Bio-Rad.

The OD₅₄₀ was measured with a Beckman DU-40 spectrophotometer.

RESULTS

Construction of *recA::lacZ* operon fusion strains

To study the regulation of transcription of the *recA* gene via detection of β -galactosidase activity, two strains were constructed in which *lacZ* expression is controlled by the *recA* promoter. One of these strains, AAC406, exhibits a RecA[−] phenotype, due to disruption of the *recA* gene by the *lacZ* insertion (Fig. 1a). The other strain, AAC407, carries a wild-type copy of *recA* downstream of the operon fusion (Fig. 1b) and therefore exhibits a RecA⁺ phenotype. In batch cultures of both types of strains, significant β -galactosidase levels were found (see below), while cultures of strains containing the *lacZ* gene in the opposite orientation with respect to the *recA* gene showed low β -galactosidase levels, comparable to the level found in the wild-type strain BD413 (data not shown).

AAC407 was constructed via a Campbell-like insertion (also termed duplication insertion) of plasmid pARA4. This type of integration results in duplication of the homologous DNA fragment on the plasmid taking part in the insertional recombination reaction. In *Deinococcus radiodurans* and *H. influenzae*, Campbell-like insertion easily leads to amplification of the inserted fragment (Masters *et al.*, 1991; Zulty & Barcak, 1993). Ten or more copies of the inserted fragment can often be detected on the chromosome. Therefore, chromosomal DNA of strain AAC407, besides being checked for integration, was also checked for amplification of the inserted fragment by Southern hybridization of *EcoRV*-digested chromosomal DNA, with the 2.3 kb *HindIII* fragment from pARA0 containing the entire *recA* gene as a probe. *EcoRV* restriction results in the formation of a fragment containing the wild-type *recA* gene, a second fragment containing the first part of the *recA::lacZ* fusion and a third fragment containing the second part of the *recA::lacZ* fusion. In the case of amplification, only the copy number of the latter fragment would increase. This increase can be visualized via hybridization with the *recA*

gene, by comparing the colour intensity of the hybridizing signals of the three fragments. The Southern blot clearly revealed that pARA4 had been integrated into the *A. calcoaceticus* chromosome. No indication, however, was found of a possible amplification of the inserted fragment in strain AAC407.

Induction of *recA* transcription by DNA-damaging agents

The effect of DNA damage on transcription of *recA*, determined via the β -galactosidase levels in the *recA::lacZ* fusion strains, was tested. In *E. coli*, and in all other bacteria in which this was tested, SOS induction was dependent on the presence of a functional RecA protein (Little & Mount, 1982; Lovett *et al.*, 1988; Venkatesh & Das, 1992; Vosman *et al.*, 1993; Zulty & Barcak, 1993). Surprisingly, this is not the case in *A. calcoaceticus*: 2 h after the addition of 4 μ g mitomycin C ml^{−1}, *recA* gene transcription was induced approximately twofold, both in AAC406 [no functional RecA; induced 2.3-fold from 980 $\pm$ 100 to 2250 $\pm$ 110 MU (mean of eight independent experiments)] and in AAC407 [RecA-proficient; induced 1.8-fold from 740 $\pm$ 70 to 1350 $\pm$ 90 MU (mean of eight independent experiments)]. Similar results were obtained with 8 μ g mitomycin C ml^{−1}, 20 μ g nalidixic acid ml^{−1}, 0.04% MMS and with UV light (254 nm, 20 J m^{−2}) as the damage-inducing agent (data not shown).

When the β -galactosidase level of cultures of strains AAC406 and AAC407 was followed with time during growth in batch culture (Fig. 2), it could be seen that both strains displayed a basic level of *recA* expression. After the addition of mitomycin C (which also negatively affected the growth of both strains), the β -galactosidase level increased approximately twofold in 2–3 h in both strains. Thus, while *A. calcoaceticus recA* expression exhibited a time-course of DNA-damage induction that was comparable to that found in other organisms, it was unique in the respect that it was independent of functional RecA.

The fact that induction of *recA* gene transcription after DNA damage was independent of the presence of functional RecA protein indicates that the expression of *A. calcoaceticus recA* is regulated in a novel, non-SOS-like fashion. In order to determine if an activator or a repressor is involved in the regulation of the expression of *A. calcoaceticus recA*, a plasmid (pARA11) was constructed that is able to replicate in *A. calcoaceticus* and that contains 1.1 kb of DNA upstream of the *recA* gene, but not the complete *recA* gene. The presence of multiple copies of the *recA* promoter might titrate a regulator from the chromosomal *recA* promoter. A lower level of β -galactosidase produced in this situation would then point towards the presence of an activator, while a β -galactosidase level comparable to that in DNA-damaged cells would indicate that a repressor is involved. pARA11 is based on the *E. coli*–*A. calcoaceticus* shuttle vector pWH1274 (Hunger *et al.*, 1990), which shows a stable copy number of between five and ten in *A. calcoaceticus* (data not shown). pARA11 contains 0.4 kb of the 5'-end of the *recA* gene and 1.1 kb of upstream sequence. This

Fig. 2. Effect of mitomycin C on growth and β -galactosidase activities of *A. calcoaceticus* strains AAC406 (a) and AAC407 (b). An overnight culture was diluted to an OD₅₄₀ of 0.1 into fresh minimal medium containing 15 μ g kanamycin ml⁻¹. After 3 h incubation at 30 °C on a rotary shaker, 4 μ g mitomycin C ml⁻¹ was added. Filled symbols, untreated culture; open symbols, mitomycin-C-treated culture; ●, ○, growth; ■, □, β -galactosidase activity. The experiments were carried out three times with essentially the same results and each curve shown is based on a representative experiment. The β -galactosidase levels given are the mean of two measurements (SD approximately 10%).

amount of upstream sequence should be sufficient to comprise all sites involved in regulation of *recA* transcription. Both pARA11 and pWH1274 were independently introduced into strains AAC406 and AAC407 and copy numbers of both plasmids were found to be comparable (data not shown). Transformants containing the plasmids in their independently replicating forms were selected by plating transformants at an ampicillin concentration of 600 μ g ml⁻¹. Although strain AAC407 itself is already resistant to 200 μ g ampicillin ml⁻¹,

transformants of this strain could be isolated by selection against higher concentrations (e.g. 600 μ g ml⁻¹) of this antibiotic. Plasmid isolations showed the presence of independently replicating pWH1274 and pARA11 in both the AAC406 and the AAC407 transformants (data not shown).

Levels of β -galactosidase activity were measured using cultures of AAC406 and AAC407 in the middle of their exponential-phase growth (OD₅₄₀ approximately 0.4) and containing pWH1274 or pARA11. The results show that when AAC406 and AAC407 each harbour pARA11, they exhibit *recA* transcription levels that are comparable to the levels found in DNA-damage-induced cells (1810 ± 130 and 1310 ± 90 MU, respectively; mean of two independent experiments). The control strains, harbouring pWH1274, showed β -galactosidase levels similar to those found in AAC406 and AAC407 without plasmids (1030 ± 100 and 680 ± 60 MU, respectively; mean of two independent experiments). This indicates that a repressor, encoded by the chromosome of *A. calcoaceticus*, is titrated by the plasmid-derived copies of the *recA* promoter, which results in a derepressed *recA::lacZ* operon fusion.

Effect of competence induction on *recA* transcription

One of the typical characteristics of competence development for natural transformation in *A. calcoaceticus* BD413 is that after dilution of an overnight culture into fresh medium, competence is induced and maximal competence is reached after 1–2 h growth at maximal growth rate (Palmen *et al.*, 1993). Chromosomal DNA, taken up via the natural transformation pathway, has to be incorporated into the recipient's chromosome in order to allow coding sequences to be expressed. Integration is facilitated by homologous recombination, a process in which RecA plays an important role. Therefore, it is conceivable that *recA* expression is also under control of the competence regulatory system, as has been observed, for instance, in *B. subtilis* (Cheo *et al.*, 1993). A first indication that transcription of the *A. calcoaceticus* *recA* gene is not influenced by the level of competence for natural transformation can be derived from the observation that in batch cultures of both strain AAC406 and strain AAC407, β -galactosidase levels do not change significantly while the cells are progressing through the consecutive stages of growth (Fig. 2), while competence of *A. calcoaceticus* is low immediately after dilution of an overnight culture and is maximal after 2 h incubation (Palmen *et al.*, 1993).

To determine more precisely the effect of the level of competence on the induction of *recA* transcription, AAC407 was grown under conditions known to give a low expression of competence. To obtain a culture with a low level of competence, AAC407 was grown in a steady-state batch culture in minimal medium. It has been shown (Palmen *et al.*, 1994) that in such a system of extended exponential growth, the transformation frequency of *A. calcoaceticus*, used as an indicator for the level of competence for natural transformation, gradually decreases

several orders of magnitude. Extended exponential growth was obtained by repeatedly diluting an *A. calcoaceticus* AAC407 culture into fresh, prewarmed minimal medium as soon as an OD₅₄₀ of 0.1 was reached. After 4 d of exponential growth, the transformation frequency and the β -galactosidase level of the culture were determined and compared to the transformation frequency and the β -galactosidase level obtained, using AAC407 at the level of maximal competence (2 h after the initial 25-fold dilution into fresh, prewarmed minimal medium). While the transformation frequencies differed 1000-fold (1×10^{-5} after extended exponential growth and 1×10^{-2} at maximal competence), the β -galactosidase levels were comparable (690 MU after extended exponential growth and 630 MU at maximal competence). This indicates that the level of competence for natural transformation of *A. calcoaceticus* has no effect on *recA* gene transcription.

To obtain a culture with a minimal level of competence, strain AAC407 was grown in a carbon-source-limited chemostat at a dilution rate of 0.1 h^{-1} (Palmen *et al.*, 1994). The transformation frequency of such a culture in steady state was 1×10^{-6} , whereas the batch culture, run in parallel, transformed at a frequency of 1×10^{-2} (i.e. a 10000-fold decrease in transformability). Determination of the β -galactosidase level in four independent samples from two independent chemostat cultures, much to our surprise, showed a mean β -galactosidase activity of 1240 (± 130) MU, i.e. comparable to the level obtained upon induction of DNA damage with MMS in batch-cultured cells (see above).

The MU normalizes enzyme activity by taking into account the amount of cells via measurement of the optical density. Cells grown in a batch culture do not necessarily have the same scattering characteristics as cells grown in a carbon-source-limited chemostat. However, cells from chemostat and from batch cultures (in minimal medium) with comparable optical densities were found to display comparable protein to optical density ratios (data not shown). Thus, the β -galactosidase levels found in the chemostat culture can be readily compared to those found in batch cultures in minimal medium (Fig. 2). Accordingly, it can be concluded that the activity of β -galactosidase in chemostat cultures (minimally competent) of AAC407 is approximately twofold higher than in batch cultures (maximally competent) [740 (± 70) MU]. Thus, it appears that *recA* transcription in the carbon-limited chemostat culture is induced to a similar extent as in batch cultures subject to DNA damage.

DISCUSSION

We have examined the regulation of the *A. calcoaceticus* *recA* gene using *lacZ* operon fusions integrated into the *A. calcoaceticus* chromosome at the position of the wild-type *recA* gene. This situation is closest to the wild-type situation, in contrast to using operon fusions located on plasmids, where multicopy effects occur and the regulatory region incorporated into the construct might be incomplete. The use of both Campbell-like and replacement recombinants made it possible to study gene

expression in the presence and absence of the functional gene product, which in the case of *RecA* is particularly important.

The regulation of the expression of the *A. calcoaceticus* *recA* gene is unique: the induction of its transcription by DNA damage is independent of the presence of functional *RecA*. The typical SOS response of *E. coli* (Little & Mount, 1982), but e.g. also of *B. subtilis* (Cheo *et al.*, 1991), *P. stutzeri* (Vosman *et al.*, 1993) and *H. influenzae* (Zulty & Barcak, 1993), is dependent on a functional *RecA* protein. In these organisms, *RecA* is required, after activation by ssDNA, for enhancement of autocleavage activity of LexA, the repressor of the *recA* gene (and other SOS-inducible genes). In agreement with this, it was recently observed that the upstream sequence of the *A. calcoaceticus* *recA* gene is not preceded by a LexA-binding motif (Gregg-Jolly & Ornston, 1994). The promoter regions of the *recA* genes of *T. ferrooxidans* (Ramesar *et al.*, 1989), *N. gonorrhoeae* (Fyfe & Davies, 1990) and *Agrobacterium tumefaciens* (Wardhan *et al.*, 1992) have also been reported to lack discernable LexA-binding sites. However, *A. calcoaceticus* is the first organism for which it has been observed that induction of *recA* by DNA damage is independent of functional *RecA*.

Our results suggest that a LexA-like protein probably is not involved in the regulation of *A. calcoaceticus* *recA* expression. In an attempt to characterize the occurrence of the SOS system in Gram-negative bacteria, Fernandez de Henestrosa *et al.* (1991) have studied the transcription of the *E. coli* *recA* gene in 30 different species. An *E. coli* *recA::lacZ* operon fusion was transferred into these bacteria on a broad-host-range plasmid and transcription levels in the absence and presence of mitomycin C were determined. It was found that in a wild-type *A. calcoaceticus* background, the *E. coli* *recA* gene is induced twofold upon treatment with mitomycin C, which is comparable to our results. The authors' conclusion that this indicates that *A. calcoaceticus* possesses a LexA-dependent SOS induction mechanism, comparable to the *E. coli* mechanism, has to be questioned on the basis of the *RecA*-independent induction, as we report in this study (and because of the absence of a LexA-binding motif upstream of the *recA* gene). These results demonstrate that at least *RecA*-deficient mutants have to be analysed before conclusions can be drawn about the presence of an SOS-like response.

There is a limited (approximately twofold) inductive response of *A. calcoaceticus* *recA* upon addition of DNA-damaging agents. In contrast, *E. coli* increases *recA* transcription 17-fold upon such a treatment (Casaregola *et al.*, 1982). Comparably low levels of *recA* induction by DNA damage have been observed in *A. vinelandii* (at most twofold; Venkatesh & Das, 1992) and *H. influenzae* (approximately threefold; Zulty & Barcak, 1993); interestingly, both are also naturally transformable Gram-negative organisms. A possible explanation for the absence of a large inductive response after addition of a DNA-damage-inducing agent may be the fact that the constitutive level of *recA* transcription in these organisms

is substantial. This would be in agreement with the observation that *recA* transcription is not induced by the competence regulatory system of *H. influenzae* (Zulty & Barcak, 1993) and *A. calcoaceticus*. Also in *P. stutzeri*, no indications have been obtained that competence for natural transformation influences *recA* expression (Vosman *et al.*, 1993; P. J. G. Rauch, unpublished results). Apparently, the constitutive level of *recA* transcription in all these Gram-negative organisms is sufficiently high to allow integration of internalized DNA.

Definite conclusions about the regulation of the expression of *A. calcoaceticus* *recA* in *E. coli*, which are beyond the framework of this investigation, cannot yet be drawn. The complementation of the *recA* genotype of *E. coli* DH5 α by the RecA enzyme from *Acinetobacter* via pARA0 may (partly) be due to a plasmid-derived promoter. LacZ-expression levels from transcriptional fusion constructs with *A. calcoaceticus* *recA* were not measurably affected by MMS, neither in *E. coli* DH5 α nor in the *recA*⁺ *lexA*⁺ strain *E. coli* AB1157. However, variations in plasmid copy number may interfere in these experiments with changes in the expression level of *recA::lacZ*.

Multiple copies of the *recA* promoter region (introduced via pARA11) appear to titrate a repressor from the chromosomal *recA* promoter. This titration effect of the *recA* promoter is not recognizable in a comparison of AAC406 and AAC407. We interpret this latter observation, however, as a consequence of differences in mRNA stability (see below). Future experiments should be aimed at identifying the postulated repressor and at analysing how its response to DNA damage is effectuated.

In species displaying LexA-dependent SOS induction [e.g. *E. coli* (Casaregola *et al.*, 1982) or *P. aeruginosa* (Horn & Ohman, 1988)], a *recA::lacZ* fusion strain has a lower level of transcription than a Campbell-like integrant containing an additional wild-type copy of *recA*. This can be explained by a titration effect of *recA* regulatory sequences on the LexA repressor, resulting in partial derepression of the *recA* promoter. This is not the case in *A. calcoaceticus*. The β -galactosidase activity of strain AAC407, under non-inducing conditions, is approximately 75 % of the level found in strain AAC406. If this difference in uninduced transcription levels would be the result of a difference in the basal levels of *recA* expression, the induced levels of transcription would be expected to be similar. However, the mitomycin C- and the pARA11-induced β -galactosidase levels that are reached in AAC407 are also lower than those in AAC406 (mitomycin C: 60 %; pARA11: 73 %). We favour the explanation that the differences in β -galactosidase levels measured between these two strains do not reflect a difference in transcription level, but a difference in stability of the mRNAs produced in both strains. Since in strain AAC406 the sequences downstream of the *recA* gene are identical to the wild-type sequences (Fig. 1), synthesis of the *recA::lacZ* mRNA is expected to terminate at the wild-type site. It is conceivable that this termination site is located downstream from the *Hind*III site, used in the cloning of the *recA* gene in pARA4. If this is the case, termination of

transcription of the *recA::lacZ* fusion in strain AAC407 probably occurs somewhere in the vector sequence present downstream of the *recA::lacZ* fusion in AAC407 (Fig. 1). This could lead to an mRNA that is less stable than the mRNA synthesized in strain AAC406. The differences in β -galactosidase levels, measured in strains AAC406 and AAC407, however, do not discount our conclusion that transcription of the *recA* gene in *A. calcoaceticus* is induced two- to threefold in cells that have been treated with DNA-damage-inducing agents, by a mechanism that is independent of the presence of functional RecA, and that competence development in *A. calcoaceticus* does not lead to increased *recA* expression.

The results obtained with the carbon-limited chemostat culture confirm the conclusion drawn from the steady-state batch culture, i.e. a less than maximal level of competence for natural transformation does not lead to decreasing levels of *recA* expression. In addition, however, the chemostat experiment shows the surprising result that carbon limitation, at a relatively low growth rate, gives rise to an increase of *recA* expression. The regulatory mechanism(s) responsible for this increase is/are still completely unknown. However, as the level of RecA in the cell is an important factor in determining the frequency at which spontaneous mutations occur (Gregg-Jolly & Ornston, 1994), this is one mechanism by which this mutation rate is coupled to the physiological state of the cells (see also Mittler & Lenski, 1990).

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