UvA-DARE (Digital Academic Repository)

Acinetobacter calcaoceticus liberates chromosomal DNA during induction of competence by cell lysis.

Palmen, R.P.C.; Hellingwerf, K.J.

DOI

[10.1007/BF01570183](https://doi.org/10.1007/BF01570183)

Publication date

1994

Published in

Current Microbiology

[Link to publication](#)

Citation for published version (APA):

Palmen, R. P. C., & Hellingwerf, K. J. (1994). Acinetobacter calcaoceticus liberates chromosomal DNA during induction of competence by cell lysis. *Current Microbiology*, 29, 1-4. <https://doi.org/10.1007/BF01570183>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Acinetobacter calcoaceticus Liberates Chromosomal DNA During Induction of Competence by Cell Lysis

Ronald Palmen, Klaas J. Hellingwerf

Department of Microbiology, E.C. Slater Institute, BioCentrum Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands

Abstract. A transformation assay was used to assay the amount of DNA present in the extracellular medium of a growing culture of *Acinetobacter calcoaceticus*. It was observed that small amounts of DNA were liberated during the entire exponential growth phase in a batch culture. Release of DNA could be fully accounted for by lysis of cells. Lysis was quantified via simultaneous measurement of β -galactosidase activity of cells and supernatant, with a strain that contained a plasmid (pAPA100) with *lacZ* under control of a constitutive β -lactamase promoter. In conclusion, no evidence could be obtained indicating that *Acinetobacter calcoaceticus* actively excretes DNA, to be used for DNA exchange.

Naturally transformable organisms are capable of taking up naked DNA, present in their environment. Subsequent incorporation of the incoming DNA into the genome allows an organism to change its genotype.

More and more organisms are being studied with respect to natural transformation, especially with respect to the role of natural transformation in pathogenesis and with respect to gene transfer in the environment. In some of these studies the transfer of markers from donor to recipient is studied in the laboratory as well as in the natural environment [1, 11, 17, 19, 20]. High-frequency transfer of markers has been observed in a number of organisms under these conditions. This has often led to the suggestion that DNA is not only actively taken up, but that these organisms may also excrete DNA into the environment [1, 2, 4, 14, 16–18]. In *Bacillus subtilis* a sequential appearance of DNase activity, followed by liberation of chromosomal as well as plasmid DNA and induction of competence, was reported by Lorenz et al. [9]. Cell lysis, investigated with a catalase assay, could not be detected in these experiments. This led the authors to conclude that DNA is actively excreted by *B. subtilis* at the time of induction of competence [9].

In the same investigation, DNA excretion was also studied in *Acinetobacter calcoaceticus*. In this organism, growth phase-dependent DNA liberation was not observed, but extracellular DNA was present in large amounts ($\pm 1 \mu\text{g DNA/ml}$) throughout the experiment. Furthermore, also for *A. calcoaceticus*, lysis was not detected during the exponential growth phase.

Previously, we have shown that *A. calcoaceticus* induces competence at the start of exponential growth [12]. If DNA would be excreted as a response to competence induction, we would expect in *A. calcoaceticus* cultures extracellular DNA to be present throughout the growth phase, just as observed by Lorenz et al. [9]. Furthermore, experiments performed by Fry et al. [2] show a high frequency of DNA exchange between *Acinetobacter* strains in the natural environment. This could indicate that exchange is mediated by a DNA excretion mechanism in *A. calcoaceticus*.

To determine whether or not *A. calcoaceticus* does indeed actively excrete DNA during induction of competence, we compared the amount of DNA liberated via lysis with the amount of DNA actually present in the growth medium at different stages of growth. For this, it is necessary to be able to accurately determine, in a batch culture, the extent of cell lysis and the extra- and intracellular DNA content. In this study lysis was quantified with a β -galactosidase-

Table 1. List of the bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype/ phenotype	Reference
<i>A. calcoaceticus</i> BD413	wild type	[6]
<i>A. calcoaceticus</i> AAC211	Kan ^R , IVL ⁻ , Trf ⁻	[12]
<i>A. calcoaceticus</i> AAC350	Kan ^R , <i>estA</i> ⁻	This study
pPJ1	Kan ^R	[15]
pWH1274	Amp ^R , Tet ^R	[5]
pNK2804	Amp ^R , <i>lacZ</i> '	[7]
pAVA213-8	Amp ^R , Kan ^R	[12]
pAPA100	Tet ^R , <i>bla::lacZ</i> '	This study

producing strain, and the amount of DNA liberated was assayed via a bioassay (i.e., via natural transformation).

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Cultures were inoculated from overnight cultures by a 1:50 dilution into 100 ml Luria Bertani medium (LB medium), after the cells were washed twice with LB [13]. Washing is necessary because the overnight culture has a relatively high extracellular DNA content (approximately 100–1000 ng DNA/ml), probably owing to increased cell lysis of stationary-phase cells. Also, inoculation of a culture from 1-day-old, colony-grown cells results in a high initial extracellular DNA concentration, which indicates that a large amount of extracellular DNA is present also in a freshly grown colony (on the order of 1 µg per colony) or that cells lyse upon transfer to fresh medium.

Determination of DNA liberated by an *Acinetobacter* culture. In pilot experiments we observed that the amount of DNA present in the supernatant of exponentially growing *Acinetobacter* cultures was below the detection limit of the fluorescence assay used by Lorenz et al. [9]. This indicated that in our experiments the DNA concentration in the supernatant was significantly lower than the 1–1.5 µg DNA/ml previously observed [9], probably owing to the inoculation with washed cells.

To be able to detect smaller amounts of DNA, we used a transformation assay to calculate the amount of DNA present in the supernatant. In this assay, strain AAC350 was used as DNA donor, and wild-type strain BD413 as recipient. AAC350 contains an *aph3* gene (encoding kanamycin resistance) from pPJ1 [15] on a *PvuII* fragment, inserted in the *EcoRV* site of the *estA* gene of *A. calcoaceticus* BD413 [8]. Samples of AAC350 collected at different stages of growth were filtered over 0.2 µm cellulose-acetate filters, and the supernatants were stored overnight at 4°C. The next day approximately 1×10^8 competent BD413 cells were resuspended in 0.5 ml filtrate and incubated for 60 min at 30°C in a shaking waterbath to allow transformation. Subsequently 20 µl DNase (5 mg/ml) was added to remove any remaining DNA. The cells were incubated for an additional 60 min to allow DNA degradation and expression of the incorporated kanamycin marker. From a calibration curve with known DNA concentrations, the amount of DNA present in the supernatant was calculated from the observed transformation frequency [13]. The assay has a detection limit of approximately 0.05 ng DNA/ml, corresponding to a transformation

frequency of approximately 5×10^{-7} . The detection limit is determined by the amount of cells used in the transformation assay. The frequency of spontaneous resistance against kanamycin is below 1×10^{-9} .

Determination of the amount of cell lysis. To determine whether or not the DNA, detected in the supernatant, is the result of an (active) excretion process, cell lysis has to be excluded as a possible source of this DNA. To be able to accurately measure cell lysis, we constructed plasmid pAPA100. It contains the promoterless *lacZ* gene of pNK2804 [7] inserted into the *ScaI* site of the *Escherichia coli*-*Acinetobacter* shuttle vector pWH1274 [5]. The *ScaI* site resides in the ampicillin resistance gene of pWH1274, which has a constitutive promoter and therefore allows constitutive expression of the *lacZ* gene after insertion. pNK2804 was restricted with *Bam*HI, and the *lacZ* gene was isolated as a 3100-bp fragment. The *Bam*HI ends were filled up with Klenow and ligated bluntly into the *ScaI* site of pWH1274. The *lacZ* gene has its own ribosome-binding site (RBS), one stopcodon upstream of the RBS and three stopcodons tailing *lacZ*. From control experiments, in which the β-galactosidase activity of the cells was determined with and without addition of tetracycline in the growth medium, it was observed that under all conditions pAPA100 is stably maintained in *Acinetobacter*. β-Galactosidase activity was assayed according to Miller [10]. In this assay, 200 µl Z-buffer (five times concentrated) was added to 800 µl filtered supernatant. The hydrolysis reaction was initiated with 200 µl ONPG solution and terminated with 500 µl 1 M Na₂CO₃. Activity of intracellular β-galactosidase was determined after washing and resuspending cells in Z-buffer. To 10–20 µl of a culture (depending on the amount of cells), 1 ml Z-buffer was added. Next, two drops of chloroform and one drop of 0.1% SDS were added, and the suspension was vigorously mixed and incubated for 15 min at 30°C. ONPG-hydrolysis was initiated and terminated as described above.

Determination of the total amount of DNA. From the extent of lysis, as determined from the β-galactosidase activity, and the amount of DNA in the cells, present at the time of sampling, one can estimate the amount of DNA liberated by lysis. The total DNA content of the cells at the time of sampling was determined via a modified Burton assay [3]. The detection limit of this assay is approximately 500 ng DNA/ml.

Results and Discussion

Liberation of DNA at different stages of growth. From the β-galactosidase activity in the cells and in the supernatant, a lysis percentage at different stages of growth was calculated (Table 2). Control experiments in which the stability of β-galactosidase in the culture supernatant was determined showed that after 5 h of incubation at 30°C the activity of the enzyme was unimpaired, indicating that the amount of enzyme in the supernatant was determined cumulatively. The DNA concentration in the supernatant also represents the total amount of extracellular DNA present at the time of sampling, and therefore equals the amount of DNA calculated to be liberated via lysis.

Table 2 summarizes the results of a representative experiment. The amount of DNA present in the supernatant, in the samples taken up to $t = 4.25$ h,

Table 2. Liberation of DNA by *Acinetobacter* strain AAC350 (pAPA100)

Time (h)	β -Galactosidase act. (Miller unit) ^a		Lysis (%)	DNA concentration (ng/ml)		
	In cells	Supernatant		In cells ^b	Lysis ^c	Transformation ^d
0	9649	496	5.1	763 $\pm$ 99	39 $\pm$ 5	24
0.75	11612	464	4.0	368 $\pm$ 99	15 $\pm$ 4	30
1.5	12093	473	3.9	1059 $\pm$ 493	41 $\pm$ 19	37
2.25	10714	413	3.9	861 $\pm$ 99	33 $\pm$ 4	39
3.25	10526	310	2.9	1552 $\pm$ 99	46 $\pm$ 3	54
4.25	9604	223	2.3	3376 $\pm$ 148	78 $\pm$ 3	86
5.25	9399	146	1.6	6482 $\pm$ 0	101 $\pm$ 0	54
6.25	12891	114	0.9	10130 $\pm$ 0	90 $\pm$ 0	31
7.25	13581	216	1.6	11510 $\pm$ 0	183 $\pm$ 0	9
8.25	15116	151	1.0	11658 $\pm$ 49	116 $\pm$ 1	10
24	18984	269	1.4	10524 $\pm$ 0	149 $\pm$ 0	65

^a The β -galactosidase activity of the supernatant was calculated as the activity normalized to the number of cells (optical density) present in the supernatant before separation.

^b Amount of DNA present in 1 ml culture, determined via a modified Burton assay [3].

^c Amount of DNA calculated to be liberated via lysis.

^d Amount of DNA present in the supernatant, determined via the transformation assay.

matches the amount of DNA calculated to be liberated by lysing cells in the culture. Thus, the DNA detected in the supernatant in the early- and mid-exponential growth phase results from DNA liberated via lysis. The correlation between the expected and measured amount of liberated DNA is lost in the samples taken during the transition to the stationary phase. During this phase, the transformation frequency (i.e., the apparent DNA concentration) decreased by a factor of 10 (Fig. 1). Part of this decrease, a factor of 1.6, can be accounted for by a reduced transformation efficiency of freshly prepared competent cells in supernatants obtained from stationary phase cultures, compared with supernatants obtained from exponential phase cultures (determined with saturating amounts of DNA: 2 μ g DNA per transformation assay; data not shown). To obtain more information about the cause of the additional reduction, we incubated an *Acinetobacter* culture with a saturating amount of DNA (2 μ g pAVA213-8 DNA/ml) and a non-saturating amount of DNA (0.1 μ g pAVA213-8 DNA/ml). The DNA concentration was followed in time with the transformation assay (Fig. 2). The transformation frequency at the start of the experiment corresponded to the amount of DNA added. Again, in both cases, the transformation frequency decreased approximately 10-fold when the cultures entered the stationary phase. This indicates that at this stage of growth, the amount of DNA present in the supernatant, after filtration, was reduced. This may be the result of DNA degradation by DNases released from lysing cells. However, attempts

Fig. 1. DNA release in a batch culture of *A. calcoaceticus* AAC350 (pAPA100), as measured via a transformation assay. Samples of a culture of the kanamycin-resistant strain AAC350 (pAPA100) were filtered with cellulose-acetate filters. To 0.5 ml filtrate approximately 1×10^8 competent BD413 cells were added and transformed as described in Palmen et al. [13]. Kanamycin-resistant transformants were selected. ○, growth; ▼, transformation frequency.

to identify DNase activity by incubating cells from the mid-exponential growth phase with plasmid DNA for 6 h at 30°C and subsequent analysis via agarose-gel electrophoresis did not reveal such activity. An alternative explanation could be that the DNA is taken up again by the cells. However, DNA uptake studies showed that only a few percent of the total amount of DNA present can be taken up by competent cells (R. Palmen, unpublished results), whereas the 10-fold

Fig. 2. Fate of a known amount of DNA in a batch culture of *A. calcoaceticus* BD413, as measured via a transformation assay. To a culture of *A. calcoaceticus* BD413, 0.1 or 2 µg pAVA213-8 plasmid DNA/ml was added at $t = 0$. Culture samples were filtered with cellulose-acetate filters. To 0.5 ml filtrate approximately 1×10^8 competent BD413 cells were added and transformed as described in Palmen et al. [13]. Kanamycin-resistant transformants were selected. Growth: ●, 0.1 µg DNA/ml added; ○, 2 µg DNA/ml added. Transformation frequency: ▼, 0.1 µg DNA/ml added; ▽, 2 µg DNA/ml added.

reduction of the transformation frequency would indicate that 90% of the total amount of DNA present had been taken up. Furthermore, the same 10-fold reduction in transformation frequency was also observed with supernatants from a culture of the transformation-deficient strain AAC211 [12]. This strain is unable to take up DNA. A remaining explanation is that cells entering the stationary phase synthesize a DNA-binding factor, thereby removing free DNA from the supernatant.

The conclusion from this study is that there is no evidence for the existence of an active excretion pathway for DNA, related to induction of competence in *A. calcoaceticus*. The DNA observed in the supernatant of *A. calcoaceticus* cultures from early- and mid-exponential growth phase must be accounted for by lysis of the cells. Furthermore, absence of lysis data, in experiments testing marker transfer via natural transformation, precludes any conclusion about an active excretion system. Also, measuring cell lysis, with catalase activity as an indicator [9], may suffer from low sensitivity, so that relatively low percentages of lysis, as observed in our studies, remain undetected.

Literature Cited

- Albritton WL, Setlow JK, Slaney L (1982) Transfer of *Haemophilus influenzae* chromosomal genes by cell-to-cell contact. *J Bacteriol* 152:1066–1070
- Fry JC, Day MJ, Williams HG (1993) Plasmid and chromosomal gene transfer by transformation in the aquatic environment. In: Balla É, Berencsi G, Szentirmai A (eds) DNA transfer and gene expression in microorganisms. Andover: Intercept Ltd, pp 111–122
- Gendimenico GJ, Bouquin PL, Trampusch KM (1988) Diphenylamine-colorimetric method for DNA assay: a shortened procedure by incubating samples at 50°C. *Anal Biochem* 173:45–48
- Hara T, Ueda S (1981) A study on the mechanism of DNA excretion from *Pseudomonas aeruginosa* KYU-1. Effect of mitomycin C on extracellular DNA production. *Agric Biol Chem* 11:2457–2461
- Hunger M, Schmucker R, Kishan V, Hillen W (1990) Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. *Gene* 87:45–51
- Juni E (1972) Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J Bacteriol* 112:917–931
- Kleckner N, Bender J, Gottesman S (1991) Uses of transposons with emphasis on Tn10. *Methods Enzymol* 204:138–180
- Kok RG, Christoffels VM, Vosman B, Hellingwerf KJ (1993) Growth-phase dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: cloning of a gene, encoding one of the esterases. *J Gen Microbiol* 139:2329–2342
- Lorenz MG, Gerjets D, Wackernagel W (1991) Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. *Arch Microbiol* 156:319–326
- Miller JH (1982) Experiments in molecular genetics. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Orrigo C, Arnaud M, Halvorson HO (1978) *Bacillus subtilis* 168 genetic transformation mediated by outgrowing spores: necessity for cell contact. *J Bacteriol* 134:973–981
- Palmen R, Vosman B, Kok RG, van der Zee JR, Hellingwerf KJ (1992) Characterization of transformation-deficient mutants of *Acinetobacter calcoaceticus*. *Mol Microbiol* 6:1747–1754
- Palmen R, Vosman B, Buijsman P, Breek CKD, Hellingwerf KJ (1993) Physiological characterization of natural transformation in *Acinetobacter calcoaceticus*. *J Gen Microbiol* 139:295–305
- Paul JH, Jeffrey WH, DeFlaun MF (1987) Dynamics of extracellular DNA in the marine environment. *Appl Environ Microbiol* 53:170–179
- Peeters BPH, de Boer JH, Bron S, Venema G (1988) Structural plasmid instability in *B. subtilis*: effect of direct and inverted repeats. *Mol Gen Genet* 212:450–458
- Reaney DC, Gowland PC, Slater JH (1983) Genetic interactions among communities. In: Slater JH, Whittenbury R, Wimpenny JWT (eds) Microbes in the natural environment. Cambridge: Cambridge University Press, pp 379–421
- Steinberg VI, Goldberg ID (1980) On the question of chromosomal gene transfer via conjugation in *Neisseria gonorrhoeae*. *J Bacteriol* 142:350–354
- Stewart GJ, Carlson CA, Ingraham JL (1983) Evidence for an active role of donor cells in natural transformation of *Pseudomonas stutzeri*. *J Bacteriol* 156:30–35
- Vakeria D, Fewson CA, Vivian A (1985) Gene transfer in *Acinetobacter calcoaceticus* NCIB8250. *FEMS Microbiol Lett* 26:141–145
- Williams HG, Day MJ, Fry JC (1992) Detecting natural transformation of *Acinetobacter calcoaceticus*, in situ natural epilithon of the River Taff. *FEMS Symp* 63:253–255