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The DNA gyrase inhibitors, nalidixic acid and oxolinic acid, prevent iron-mediated repression of catechol siderophore synthesis in *Azotobacter vinelandii*

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Summary. Low concentrations of nalidixic acid and oxolinic acid that were just inhibitory to *Azotobacter vinelandii* growth promoted the production of the catechol siderophores azotochelin and aminochelin, in the presence of normally repressive concentrations of Fe^{3+}. There was a limited effect on the pyoverdin siderophore, azotobactin, where low concentrations of Fe^{3+} were rendered less repressive, but the repression by higher concentrations of Fe^{3+} was normal. These drugs did not induce high-molecular-mass iron-repressible outer-membrane proteins and similar effects on the regulation of catechol siderophore synthesis were not produced by novobiocin, coumermycin, or ethidium bromide. The timing of nalidixic acid and Fe^{3+} addition to iron-limited cells was critical. Nalidixic acid had to be added before iron-repression of catechol siderophore synthesis and before the onset of iron-sufficient growth. Continued production of the catechol siderophores, however, was not due to interference with normal iron uptake. These data indicated that nalidixic acid prevented normal iron-repression of catechol siderophore synthesis but could not reverse iron repression once it had ocurred. The possible roles of DNA gyrase activity in the regulation of catechol siderophore synthesis is discussed.

Key words: *Azotobacter vinelandii* — Catechols — Siderophores — Regulation — Nalidixic acid

Introduction

Azotobacter vinelandii, an obligate aerobe capable of nitrogen fixation, produces a number of siderophores for iron accumulation (Knosp et al. 1984; Page and von Tigerstrom 1988). These include a yellow-green fluorescent peptide siderophoe in the pyoverdin family (azotobactin) (Demange et al. 1986), *N,N'*-bis(2,3-dihydroxybenzoyl)-L-lysine (azotochelin) (Corbin and Bulen 1969), and 2,3-dihydroxybenzoylputrescine (aminochelin) (Page and von Tigerstrom 1988). In addition to these iron-repressible compounds, these cells also produce 2,3-dihydroxybenzoic constitutively to promote iron solubilization and low-affinity iron uptake (Page and Huyer 1984).

An unusual feature of the regulation of these Fe^{3+}-uptake ligands in *A. vinelandii* is the sequential repression of siderophore synthesis by iron (Page and Huyer 1984). Azotobactin production and azotobactin-mediated ^{55}Fe uptake is tightly regulated by iron availability and is repressed by 2.5–3.0 μM Fe^{3+} (Page and Grant 1988; Page and Huyer 1984; Page and von Tigerstrom 1988). At these concentrations of Fe^{3+}, however, the catechol siderophores continue to be formed and function in ^{55}Fe uptake. Aminochelin is produced at a constant proportion of the total catechols and at 6.0–7.5 μM Fe^{3+} both aminochelin and azotochelin are repressed coordinately, leaving 2,3-dihydroxybenzoic acid as the sole extracellular participant in iron uptake (Page and von Tigerstrom 1988). High-molecular-mass iron-repressible outer-membrane proteins also have been identified as part of the high-affinity iron-uptake system and there also is evidence of a differential effect of iron on their synthesis (Page and Huyer 1984; Page and von Tigerstrom 1982).

The regulation of iron-repressible genes in *Escherichia coli* has been attributed to a repressor protein (Fur) which in combination with the corepressor Fe^{2+} acts at specific promotors (Bagg and Neilands 1987; De Lorenzo et al. 1987). Assuming

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a similar form of regulation exists in *A. vinelandii*, sequential regulation could be achieved through variations in promotor affinity for the repressor complex. However, variations in DNA superhelical tension can also increase or decrease gene expression and influence operator-repressor interactions (Drlica 1984; Gellert 1981; Sanzey 1979; Smith 1981; Smith et al. 1978). The degree of DNA supercoiling in *E. coli* is determined by a balance between DNA-winding (DNA gyrase; topoisomerase II) and DNA-relaxing (topoisomerase I) activities (Menzel and Gellert 1983). This present study was, therefore, initiated to determine if DNA gyrase inhibitors would affect the synthesis of siderophores in *A. vinelandii* as a first step in assessing the role of DNA supercoiling in the regulation of iron-repressible genes.

Materials and methods

Bacteria and growth conditions. The studies used the capsule-negative strain UW of *A. vinelandii* OP. The cells were grown in Burk medium, pH 7.2, containing 1% (wt/vol) glucose and 1.1 g ammonium acetate/l (Page and Sadoff 1976). Iron-limited medium contained no added iron and iron availability was varied by the addition of ferric citrate. DNA gyrase inhibitors were filter-sterilized and added to sterile medium. All drugs were aqueous solutions except coumermycin which was dissolved in sterile dimethylsulfoxide. There was no effect of dimethylsulfoxide in the concentrations used ($\leq$0.5%, vol/vol) on the growth of *A. vinelandii*.

The inoculum for each study was pregrown for 48–72 h on Burk medium slants, containing 18 μM ferrous sulfate and 1.8% agar (wt/vol), by which time the cells were iron-limited and fluorescent green (Page and Huyer 1984). The inoculum was then prepared and added as described previously (Page and Huyer 1984). The flasks were incubated for 22–24 h at 30° C with gyratory shaking at 225 rpm.

Siderophore analysis. Catechol concentrations in culture supernatant fluids were assayed by the Barnum method, using 2,3-dihydroxybenzoic acid as a standard (Barnum 1977). Azotobactin was estimated by its absorbance at 380 nm in acidified culture supernatant fluids, as described previously (Knosp et al. 1984).

Azotochelin and 2,3-dihydroxybenzoic acid were extracted from acidified culture supernatant fluids using ethyl-acetate (Corbin and Bulen 1969). The ethyl-acetate-soluble catechols were identified after silica gel G thin-layer chromatography (TLC). Chromatograms were developed in a benzene/acetic acid/water (125:72:3, vol/vol) solvent system and sprayed with $FeCl_3$/bipyridyl (Corbin and Bulen 1969; Page and Huyer 1984). Aminochelin remained in the aqueous phase after ethyl-acetate extraction (Page and von Tigerstrom 1988) and was quantified by the Barnum assay (Barnum 1977).

Cell fractionation. Outer membranes were isolated by the sodium lauroyl sarcosinate procedure described previously (Page and Huyer 1984). Analysis of the proteins present in these membranes was by polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol, using the apparatus and conditions of Page and von Tigerstrom (1982).

Cell yield was estimated by protein assay and iron content was determined after digestion of the cells in hot perchloric acid (Page and Huyer 1984). The DNA content of the cells was determined after fractionation with cold 5% (wt/vol) trichloroacetic acid (0° C, 15 min) followed by hot 5% trichloroacetic acid (90° C, 20 min) and the colorimetric assay of Giles and Meyers (1965).

All values reported are the means of at least duplicate assays.

Results

Effect of nalidixic acid

After a 24-h incubation of a culture containing 10 μM Fe^{3+} and 20 mg ml^{-1} nalidixic acid (NA) there was a 14% decrease in cell protein yield and an 18% decrease in μg DNA (mg cell protein)$^{-1}$ compared to the iron-containing control culture. The control culture supernatant fluid contained only 2 μg catechol (mg protein)$^{-1}$, whereas the NA-treated culture contained 20 μg catechol (mg protein)$^{-1}$. NA at 10 and 15 μg ml^{-1} had an almost identical effect as that observed for 20 μg ml^{-1} NA. The amount of catechol produced was affected by the amount of available Fe^{3+} (Fig. 1). Without added Fe^{3+}, NA had no effect on catechol production. With the addition of 1–5 μM

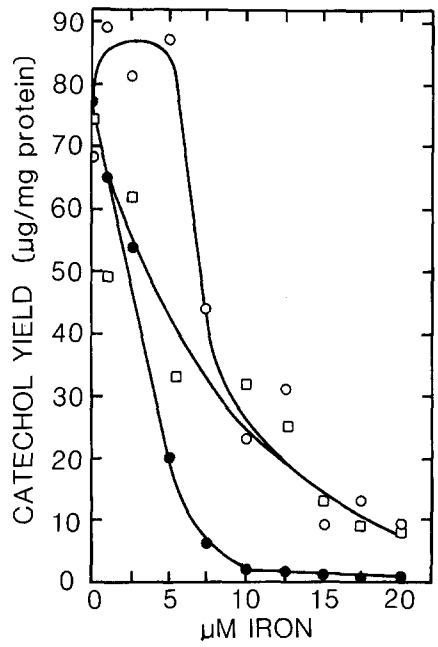

Fig. 1. Production of catechols in the presence of normally repressive iron concentrations. The yield of catechol produced per mg cell protein in cultures with varied iron contents, after a 24-h incubation, in the presence of 20 μg ml^{-1} NA (○), 2 μg ml^{-1} OA (□) or without drug addition (●) is shown

Fe^{3+}, catechol was hyperproduced at 85 μg (mg protein)$^{-1}$ (1.25 times the iron-limited control). There was a steady decline in catechol production with increased Fe^{3+} availability, but even at 20 μM Fe^{3+}, catechol was present at 9 μg (mg protein)$^{-1}$, 30-fold greater than the corresponding control.

Ethyl-acetate extraction of the culture supernatant fluids and TLC showed that the NA-treated cultures all contained azotochelin and 2,3-dihydroxybenzoic acid. The sizes of the TLC spots indicated that the latter was hyperproduced at 1 μM Fe^{3+}, then remained at a minor and constant concentration at higher Fe^{3+} values. The azotochelin spot appeared to vary in concentration in parallel with the results shown in Fig. 1. The aqueous phase of these ethyl-acetate-extracted culture supernatant fluids contained aminochelin, at a constant 23% ± 3% of the total catechol value. In the control culture supernatant fluids, azotochelin and aminochelin were present and repressed coordinately up to 7.5 μM Fe^{3+}. Aminochelin constituted approximately 25% ± 2% of the total catechol value and 2,3-dihydroxybenzoic acid appeared as described for the NA-treated cultures. Therefore, NA treatment was promoting the synthesis and excretion of both catechol siderophores, even in the presence of normally repressive Fe^{3+} concentrations.

Effect on azotobactin production

There was a limited effect of NA on the control of azotobactin repression by Fe^{3+}. There was no hyperproduction of azotobactin relative to the iron-limited control. In the control culture, the initial azotobactin level [2.2 A_{380} units (mg protein)$^{-1}$] decreased by 27% when 1 μM Fe^{3+} was present. With 10–20 μg NA ml^{-1} and 1 μM Fe^{3+}, there was no decrease in azotobactin production. At 2.5 μM Fe^{3+}, the control azotobactin yield decreased 80% compared to the iron-limited control, but with NA treatment the decrease was only 50%. At 5 μM Fe^{3+}, however, there was no sparing effect of NA and all cultures showed complete repression of azotobactin.

Appearance of outer membranes

Analysis of outer membranes from cells grown with 10, 15 and 20 μM Fe^{3+} showed the absence of high-molecular-mass iron-repressible proteins and showed that treatment with 20 μg NA ml^{-1} did not induce or permit the synthesis of these proteins in the presence of excess Fe^{3+} (data not shown).

Other DNA gyrase inhibitors

Oxolinic acid (OA) at 2 μg ml^{-1} inhibited *A. vinelandii* growth yield 15% after a 24-h incubation and, like NA, prevented the complete repression of azotochelin and aminochelin at > 10 mM Fe^{3+} (Fig. 1). At this OA concentration there was no hyperproduction of catechol, but Fe^{3+} was not as repressive in the presence of OA. At 1 μg ml^{-1} OA-treated cultures were identical to the controls. OA at 3 μg ml^{-1} caused 84% inhibition of growth but appeared to allow hyperproduction of the catechol siderophores at 1 and 2.5 μM Fe^{3+} [94 and 106 μg catechol (mg protein)$^{-1}$, respectively]. With 3 μg OA ml^{-1} and ≥ 5 μM Fe^{3+}, the yield of catechol duplicated the values shown for 2 μg OA ml^{-1} in Fig. 1. OA also affected azotobactin production as reported for NA and failed to induce the production of iron-repressible outer-membrane proteins in the presence of excess Fe^{3+} (data not shown).

The other DNA gyrase inhibitors novobiocin (7.5–150 μg ml^{-1}) and coumermycin (1–20 μg ml^{-1}) did not effect siderophore production under these conditions. Drug concentrations which gave 15%–20% inhibition of growth were examined in detail, but no similar effects were observed (data not shown). Similarly, ethidium bromide at 0.3–0.75 μg ml^{-1} did not effect siderophore production by *A. vinelandii*.

Timing of NA addition

When *A. vinelandii* was pregrown in iron-sufficient medium and then treated with NA or OA there was no enhancement of catechol synthesis. The effect of these drugs was only observed when Fe^{3+} and NA or OA were added to iron-limited cells.

A closer examination showed that NA had to be added not later than 3 h after Fe^{3+} addition. This period of susceptibility to NA corresponded to a lag phase before the rapid growth of the iron-sufficient culture and prior to repression of siderophore synthesis (Fig. 2). The growth curves, with or without NA treatment, were almost identical in the first 10 h of incubation. Typically for *Azotobacter* growth on ferric citrate (Page 1987), the Fe^{3+} initially available rapidly became cell-

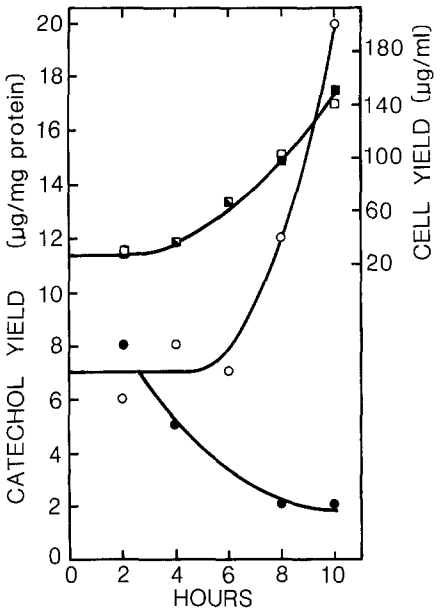

Fig. 2. Comparison of initial growth characteristics with and without addition of 20 µg ml^{-1} NA. Cell growth, measured as µg protein ml^{-1}, with (□) and without (■) NA and catechol yield with (○) and without (●) NA addition are shown. NA and 10 µg M ferric citrate were added at time zero to cultures inoculated with iron-limited cells then sampled over 10 h as shown

associated in both cultures. At 2 h, 92% [33.2 µg Fe^{3+} (mg protein)$^{-1}$] and 79% [24.8 µg Fe^{3+} (mg protein)$^{-1}$] of the initial iron was cell-associated in the control and NA-treated cultures, respectively. With continued cell growth the values of Fe^{3+} (cell protein)$^{-1}$ decreased, but the values of both the control and NA-treated cultures were characteristic of iron-sufficient cells [4.5–5.4 µg Fe^{3+} (mg protein)$^{-1}$ and 3.0–4.0 µg Fe^{3+} (mg protein)$^{-1}$ at 10 h and 24 h, respectively]. By comparison, the iron-limited control culture contained 0.6 µg Fe^{3+} (mg protein)$^{-1}$ after 10–24 h. Therefore, NA did not appear to enhance catechol siderophore production by generating iron-limited cells.

Discussion

The effect of low concentrations of NA or OA on *A. vinelandii* appears to be very subtle, affecting the iron regulation of catechol siderophore synthesis without severe inhibition of growth. These drugs do not interfere with iron uptake and, therefore, are permitting siderophore production in the presence of normally repressive external and internal iron concentrations. The results indicate that NA and OA have a greater effect on catechol siderophore synthesis than on azotobactin synthesis and suggest that DNA superhelical density may be more important in the iron regulation of the catechol siderophores than the pyoverdin siderophore azotobactin.

Both OA and NA can decrease DNA supercoiling in whole cells of *E. coli* (Drlica 1984; Gellert 1981; Gellert et al. 1977) which can have a weaking effect on repressor:promotor interaction (Sanzey 1979; Smith 1981; Smith et al. 1978). Differences in the primary sequence of the operator region and the presence of pseudo-operators may further determine the degree to which supercoiling affects the strength of repressor binding (Whitson et al. 1987). However, in cases where DNA supercoiling has been implicated in gene regulation, the drugs that affect the B subunit of DNA gyrase (Gellert et al. 1976) are also as effective as OA or NA (Drlica 1984; Sanzey 1979; Smith et al. 1978). In the case of catechol siderophore regulation, however, novobiocin and coumermycin were ineffective substitutes for OA or NA.

Low concentrations of OA or NA also promote the formation of stable (complexes between gyrase A subunit and DNA) (Gellert et al. 1980; Gellert et al. 1977; Snyder and Drlica 1979). These complexes are not formed with novobiocin or coumermycin (Drlica 1984; Gellert 1981). This differential effect suggests that drug-induced binding of the DNA gyrase to *A. vinelandii* DNA may be important in the observed abortive iron regulation of catechol siderophore synthesis. Drug-induced DNA-gyrase binding occurs at approximately 45 sites per *E. coli* genome, but not all sites bind DNA gyrase with equal affinity (Gellert et al. 1980; Snyder and Drlica 1979). Thus DNA-gyrase complexes may have a differential effect on domains of the bacterial chromosome without inducing massive changes in DNA superhelical density (Manes et al. 1983; Snyder and Drlica 1979).

The timing of NA addition to *A. vinelandii* was found to be critical for abortive iron regulation: NA prevented iron repression but did not reverse iron repression once it had occurred. Also, Fe^{3+} appeared to be competitive with NA, such that higher external Fe^{3+} resulted in lower yields of catechol, although siderophore synthesis was turned on at all concentrations of Fe^{3+} tested. A possible explanation for these results, although entirely speculative and undoubted simplistic, forms the basis for further experimental work. As proposed for iron regulation of catechol siderophore synthesis, the formation of the (critical) gy-

rase-NA-DNA complex may also depend on DNA superhelical density. DNA gyrase binding to DNA has been shown to be stronger when the DNA is linear or relaxed (Drlica 1984; Morrison et al. 1980). Therefore, if Fe^{3+} addition to iron-limited cells promotes DNA supercoiling, it could appear to have a competitive effect on the NA-induced phenomenon. Significantly, Balke and Gralla (1987) recently showed that DNA supercoiling in *E. coli* cells decreased as a result of nutrient starvation.

Another mechanism of NA and OA action that must be considered is based on fact that these drugs can act as chelators of divalent ions, through the 4-oxo-3-carboxylic acid moiety common to both compounds (Crumplin et al. 1980). Thus NA action may be unrelated to DNA supercoiling but may be related to internal competition of NA and Fur for Fe^{2+}. Work is continuing with *A. vinelandii* to test ideas developed in this discussion, to determine the role of NA and DNA supercoiling in iron-regulated gene expression.

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