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Dynamics of multigene expression during catabolic adaptation of *Ralstonia eutropha* JMP134 (pJP4) to the herbicide 2,4-dichlorophenoxyacetate

Johan H. J. Leveau,† Franziska König, Hanspeter Füchslin, Christoph Werlen and Jan Roelof van der Meer*

Swiss Federal Institute for Environmental Science and Technology (EAWAG) and Swiss Federal Institute for Technology (ETH), Ueberlandstrasse 133, CH-8600 Dübendorf, Switzerland.

Summary

***Ralstonia eutropha* JMP134 carries a 22 kb DNA region on plasmid pJP4 necessary for the degradation of 2,4-D (2,4-dichlorophenoxyacetate). In this study, expression of the 2,4-D pathway genes (designated *tfd*) upon exposure to different concentrations of 2,4-D was measured at a detailed timescale in chemostat-grown *R. eutropha* cultures. A sharp increase in mRNA levels for *tfdA*, *tfdCDEF-B*, *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$-B$_{II}$* and *tfdK* was detected between 2 and 13 min after exposure to 2,4-D. This response time was not dependent on the 2,4-D concentration. The genes *tfdA*, *tfdCD* and *tfdD$_{II}$C$_{II}$* were expressed immediately upon induction, whereas *tfdB*, *tfdB$_{II}$* and *tfdK* mRNAs could be detected only around 10 min later. The number of *tfd* mRNA transcripts per cell was estimated to be around 200–500 during maximal expression, after which they decreased to between 1 and 30 depending on the 2,4-D concentration used for induction. Unlike the mRNAs, the specific activity of the 2,4-D pathway enzyme chlorocatechol 1,2-dioxygenase did not increase sharply but accumulated to a steady-state plateau, which was dependent on the 2,4-D concentration in the medium. At 1 mM 2,4-D, several oscillations in mRNA levels were observed before steady-state expression was reached, which was caused by transient accumulation of the first pathway intermediate, 2,4-dichlorophenol, to toxic concentrations. Expression of *tfdR* and *tfdS*, the (identical) regulatory genes for the *tfd* pathway remained low and essentially unchanged during the entire adaptation phase.**

Received 9 March, 1999; revised 23 April, 1999; accepted 26 April, 1999. †Present address: Department of Plant and Microbial Biology, University of California at Berkeley, USA. *For correspondence. E-mail vdmeer@eawag.ch; Tel. (+41) 1 823 5438; Fax (+41) 1 823 5547.

Introduction

Plasmid pJP4 (Don and Pemberton, 1985) enables its bacterial host strain *Ralstonia eutropha* JMP134 (Don and Pemberton, 1981) to use 2,4-dichlorophenoxyacetate (2,4-D) as a sole source of carbon and energy. A 22 kb DNA fragment carries the genetic information for the 2,4-D pathway (Fig. 1A). Characteristic of the 2,4-D (*tfd*) pathway gene organization are its relatively recent evolutionary formation (Leveau and van der Meer, 1997) and its mosaic-like structure, being composed of DNA fragments of different bacterial origins (Fulthorpe *et al*., 1995). Among the first identified genes necessary for 2,4-D degradation were *tfdA* (Streber *et al*., 1987), *tfdB* and *tfdCDEF* (Don *et al*., 1985; Perkins *et al*., 1990). Their products catalyse the sequential conversion of 2,4-D into 3-oxoadipate via 2,4-dichlorophenol (2,4-DCP) and 3,5-dichlorocatechol (3,5-DCC) (Perkins *et al*., 1990; Fukumori and Hausinger, 1993a,b; Farhana and New, 1997) (Fig. 1B). The 22 kb fragment of pJP4 carries an additional set of genes, tentatively named *tfdD$_{II}$C$_{II}$E$_{II}$F$_{II}$-B$_{II}$* (Fig. 1A), based on their similarities to *tfdCDEF* and *tfdB* (ranging between 15% and 62% at the amino acid level) (Matrubutham and Harker, 1994; van der Meer and Leveau, 1997). However, their ability to encode a complete pathway for the conversion of 2,4-DCP to 3-oxoadipate has not yet been established. Another newly identified gene on pJP4 is *tfdK*, which encodes a protein facilitating 2,4-D transport (Leveau *et al*., 1998).

Specific enzyme activities for 2,4-D degradation are induced when *R. eutropha* JMP134 (pJP4) is cultivated on 2,4-D (Pieper *et al*., 1988). Induction of *tfd* gene expression is dependent on two identical but divergently oriented regulatory genes (*tfdR* and *tfdS*), also harboured on plasmid pJP4 (Matrubutham and Harker, 1994; You and Ghosal, 1995; Leveau and van der Meer, 1997). TfdR/S belongs to the family of LysR-type transcriptional activators (Henikoff *et al*., 1988; Schell, 1993) and binds to specific sites upstream of the *tfdA, tfdC* and *tfdD$_{II}$* promoters (Matrubutham and Harker, 1994; Leveau and van der Meer, 1996). In the presence of 2,4-dichloromuconate (Filer and Harker, 1997) or 2-chloromuconate (McFall *et al*., 1997), TfdR/S is able to activate transcription from its target promoters. Both compounds are formed as intermediates during the degradation of 2,4-D (Fig. 1B) and 3-chlorobenzoate respectively (for which the *tfdCDEF* genes are also essential). The role

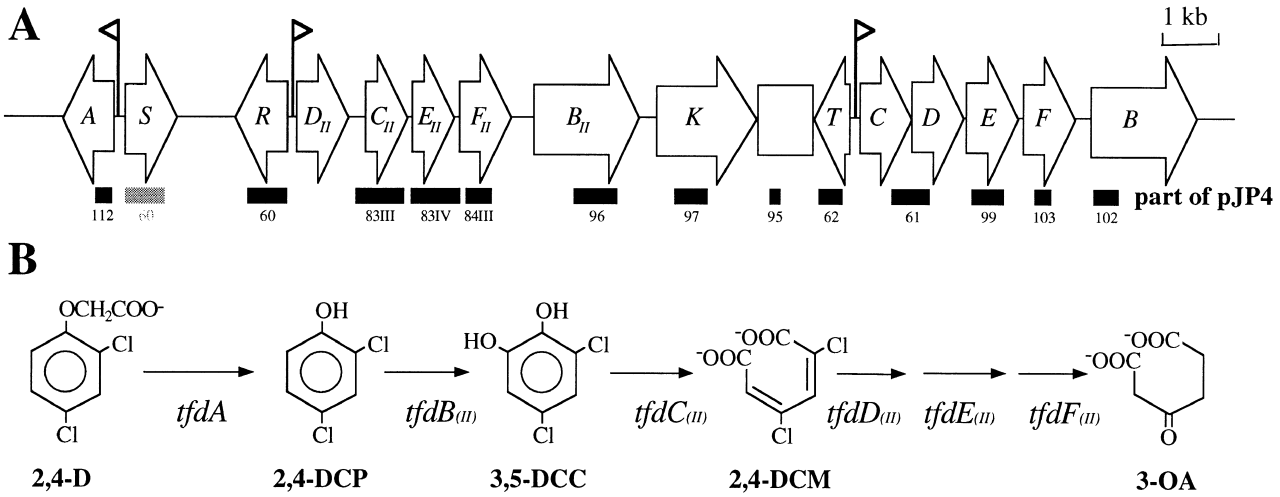

Fig. 1. A. Genetic organization of the *tfd* genes on plasmid pJP4. The orientation and sizes of the genes are indicated by arrows; the solid line is non-coding pJP4 DNA. The rectangle between *tfdK* and *tfdT* represents the IS element ISJP4. Black boxes below the genes indicate locations and sizes of the DNA fragments used for the synthesis of sense and antisense RNA probes; the numbers refer to the corresponding pCBA plasmid constructs (see *Experimental procedures*). Arrows point to the location and direction of known TfdR/S binding sites.
B. 2,4-D catabolic pathway of *R. eutropha* JMP134 (pJP4). Below each conversion step, the gene that encodes the responsible enzyme is indicated (2,4-D, 2,4-dichlorophenoxy-acetate; 2,4-DCP, 2,4-dichlorophenol; 3,5-DCC, 3,5-dichlorocatechol; 2,4-DCM, 2,4-dichloromuconate; 3-OA, 3-oxoadipate). 3-OA is further converted to intermediates of the tricarboxylate cycle by chromosomally encoded enzymes.

of 2-chloromuconate as an effector of mRNA synthesis has been demonstrated *in vitro* with the purified regulatory protein ClcR (which is closely related to TfdR) (McFall *et al*., 1997). A third regulatory gene on pJP4, *tfdT*, very similar to *tfdR* and *tfdS*, does not encode a functional protein, as it is insertionally inactivated by the element ISJP4 (Leveau and van der Meer, 1997).

The objective of this study was to investigate the expression of the *tfd* genes upon exposure of *R. eutropha* JMP134 to 2,4-D. For this purpose, we studied the transient response of *tfd* gene expression and changes in 2,4-D degradation activity (concentrations of 2,4-D, 2,4-DCP and 3,5-DCC, activity of chlorocatechol 1,2-dioxygenase, biomass) during adaptation of chemostat-grown JMP134 cells exposed to different concentrations of 2,4-D. We show that the *tfd* genes are expressed in concert from promoters present *in cis* with respect to *tfdR* (*tfdS*) and from a promoter located *in trans*. However, significantly later expression of the most distal genes in the *tfd* operons can result in unbalanced cellular physiology. Finally, we demonstrate that, in addition to expression of the previously described *tfdA*, *tfdB*, *tfdCDEF* and *tfdK* genes, the $tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ genes are also expressed in the presence of 2,4-D.

Results

Immediate expression of the tfd *genes in response to 2,4-D*

Chemostat cultures of *R. eutropha* JMP134 (pJP4) were grown to steady state on 20 mM fructose at a growth rate of 0.05 h^{-1} (13.9 h doubling time). During growth on fructose (i.e. uninduced conditions), relative mRNA levels for the *tfd* genes were around 1 or less than 1 molecule of each *tfd* mRNA species for an average *R. eutropha* cell (Table 1). After the addition of 2,4-D to 0.01, 0.1 or 1 mM, all *tfd* genes became clearly induced (Fig. 2), except for *tfdR* (*tfdS*) and *tfdT* (not shown). For highly expressed genes such as *tfdC* or *tfdA*, peak levels up to 400 mRNA molecules per cell were reached, whereas for others (e.g. $tfdE_{II}$, $tfdF_{II}$ or *tfdK*), mRNA levels did not reach above 100 (Table 1). Expression of the second *tfd* cluster (i.e. $tfdD_{II}$ to *tfdK*) was also activated. After 1 h (for 10 μM and 0.1 mM 2,4-D), *tfd* mRNA levels decreased again to lower values and remained more or less constant afterwards (tested up to 180 h after shifting). Hybridization of total RNAs with a probe for the 16S rRNA revealed similar expression levels during all phases, indicating that the activation of the *tfd* genes is a specific response to the addition of 2,4-D (not shown).

The detailed timescale of mRNA sampling allowed us to observe small differences in the accumulation of *tfd* transcripts. For example, *tfdA* mRNA was detected up to 10 min earlier than that of *tfdB* or *tfdK* (Fig. 3A). Of all *tfd* genes, *tfdA*, *tfdCD* and $tfdC_{II}$ were expressed the fastest, whereas accumulation of *tfdE*, *tfdF*, $tfdE_{II}$ or $tfdF_{II}$ mRNA was detected about 5 min later. Interestingly, the maximum levels of mRNA did not show significant differences between shifts performed at 1 mM or 0.1 mM 2,4-D (Table 1). However, the duration of maximal mRNA expression after the 1 mM shift was prolonged (Fig. 3).

Table 1. Characteristics of *tfd* gene expression.

Gene	Uninduced mRNA level[a]	Steady-state and peak mRNA levels		
		1 mM 2,4-D	0.1 mM	10 μM
tfdA	0.30 ± 0.21[b]	16.6 ± 3.5 (220 ± 28)	7.08 ± 1.34 (237 ± 17)	1.67 ± 0.78[c] (112 ± 15)
tfdR (tfdS)	1.86 ± 1.58	1.16 ± 0.60	0.36 ± 0.06	NA[d]
$tfdC_{II}$	0.61 ± 0.58	8.86 ± 4.41 (198 ± 51)	1.60 ± 1.63[c] (208 ± 7)	1.40 ± 0.89[e] (109 ± 23)
$tfdE_{II}$	0.42 ± 0.39	6.2 ± 2.4 (63 ± 14)	1.60 ± 0.77[c] (89 ± 19)	NA
$tfdF_{II}$	1.06 ± 0.24	2.07 ± 0.6 (33 ± 2)	NA	NA
$tfdB_{II}$	0.30 ± 0.19	14.1 ± 2.4 (130 ± 24)	3.7 ± 1.07 (116 ± 20)	NA
tfdK	0.87 ± 0.27	29.2 ± 10.3 (90 ± 34)	1.95 ± 0.65[c] (65 ± 13)	0.95 ± 0.19[e] (36.6 ± 6.8)
tfdCD	0.82 ± 0.74	4.26 ± 1.09 (306 ± 197)	1.23 ± 0.45[c] (382 ± 63)	0.46 ± 0.15[c] (140 ± 4.9)
tfdE	0.46 ± 0.32	10.6 ± 7.66 (310 ± 177)	1.51 ± 0.25[c] (207 ± 76)	NA
tfdF	0.93 ± 0.50	1.88 ± 0.99 (82 ± 34)	0.44 ± 0.12[e] (143)	NA
tfdB	1.80 ± 1.72	8.5 ± 3.1 (98 ± 22)	1.31 ± 0.30[c] (132 ± 17)	NA

a. Uninduced values are averaged from all pulsed shifts (i.e. seven experiments at the three different 2,4-D concentrations).
b. Values given are average relative mRNA levels ± average deviation during steady state and at the initial peak maxima (values within brackets). Average relative mRNA levels can be interpreted as the number of mRNA copies for an average cell.
c. Significantly different from the uninduced values within the same experiment.
d. NA, not analysed.
e. Not significantly different from the uninduced values within the same experiment.

After the 10 μM 2,4-D shift, however, the peak height of mRNA accumulation was also lower (Table 1). In general, peak levels were lower for transcripts from the genes more distal to the identified TfdR/S binding site (e.g. *tfdCD* versus *tfdF* or *tfdB*) (Fig. 2).

Northern analysis of the tfd *transcripts*

Northern analysis of *tfd* transcripts in total RNAs extracted from the continuously grown cells of *R. eutropha* also showed very abundant and specific induction upon exposure

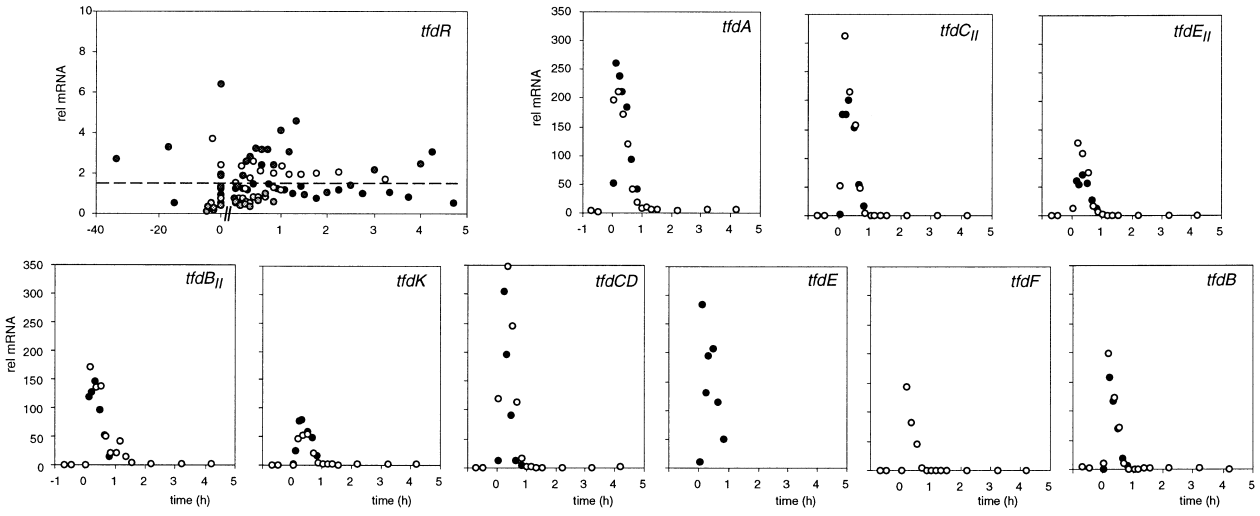

Fig. 2. Differences in the response of *tfd* gene induction to 2,4-D at a 0.1 mM pulsed shift. Shown are changes in mRNA levels during the first 5 h after the pulsed shift (at time = 0) of most *tfd* genes, except $tfdD_{II}$, which could not be assessed by the biotin-labelled probes, and $tfdF_{II}$, which was not measured in the 0.1 mM pulsed shift. Note the different axis for the relative mRNA concentrations of *tfdR* (*tfdS*). Open and closed symbols indicate data from two independent experiments. Data for *tfdR* were composed from all (6) pulsed shift experiments; the dotted line points to the mean transcript level.

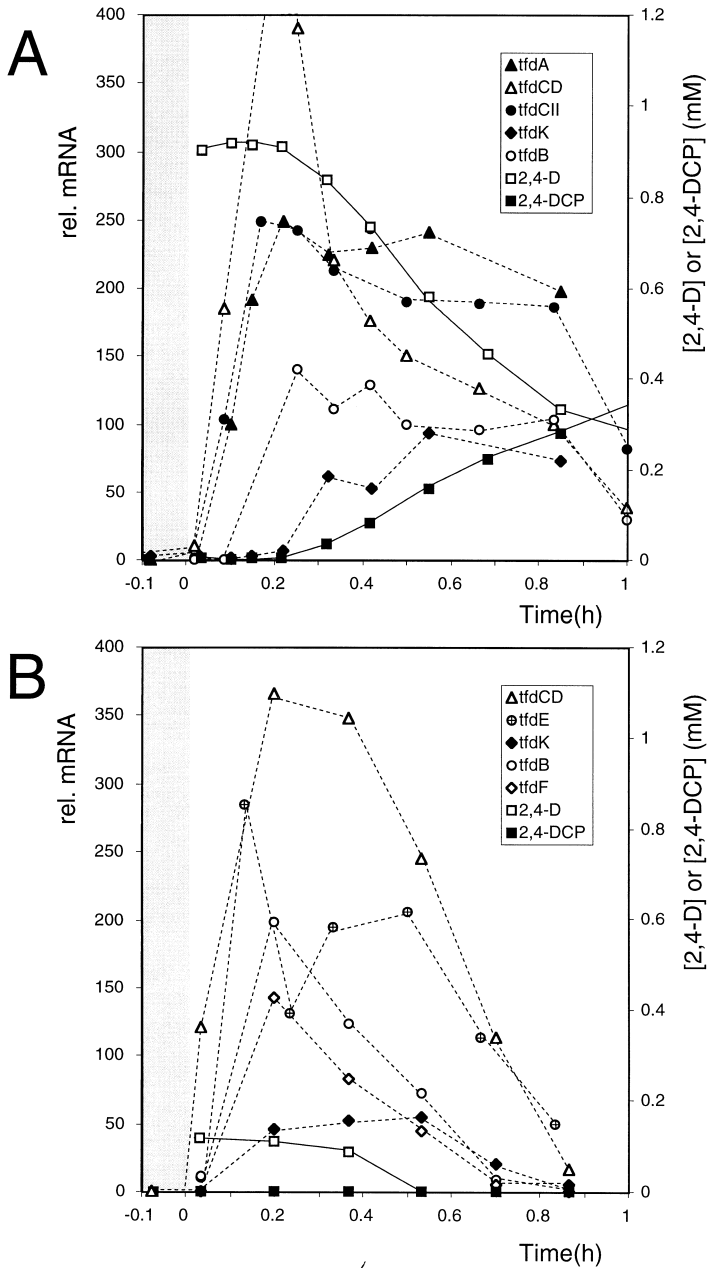

Fig. 3. Dynamics of *tfd* gene expression and the changes in 2,4-D and 2,4-DCP concentrations during the first hour after the shift.
A. Data from the 1 mM shift.
B. Data from the 0.1 mM shift.
Data in this figure were taken from one experiment each (i.e. no average values). Note the distinct delay in mRNA appearance of *tfdB* and *tfdK*.

to 2,4-D (Fig. 4). The finding that no specific *tfd* transcripts were detected in total RNA extracts from fructose-grown steady-state cells (not shown) was consistent with the results of the dot-blot hybridizations. Probing with a fragment of the *tfdA* gene clearly indicated a transcript of around 2 kb, which is in agreement with the organization of *tfdA* as a single transcribed gene (Fig. 1). Blots hybridized with probes for *tfdCD* or *tfdE* indicated transcripts at 4 kb and 2 kb. The 4 kb transcript very probably encompasses the *tfdCDEF* genes, starting from the TfdR/S-regulated promoter upstream of the *tfdC* gene (Perkins *et al*., 1990; Leveau and van der Meer, 1997). The 2 kb band seen with a *tfdCD* probe and with a *tfdE* probe could be derived from the same *tfdCDEF* mRNA species, specifically cleaved between *tfdD* and *tfdE*. Blots hybridized with a *tfdB* probe indicated a weak 6 kb and a strong 2.4 kb transcript. The 2.4 kb transcript very probably encompasses the *tfdB* gene itself. The 6 kb transcript could be formed as a long mRNA starting from the *tfdC* gene and continuing throughout *tfdB*. Hybridization with the $tfdC_{II}$ gene showed a weak transcript around 4 kb (probably encompassing $tfdD_{II}C_{II}E_{II}F_{II}$) and stronger ones at 2.4 kb and 1.4 kb, which might be specifically cleaved or degraded mRNAs. No indication was found of

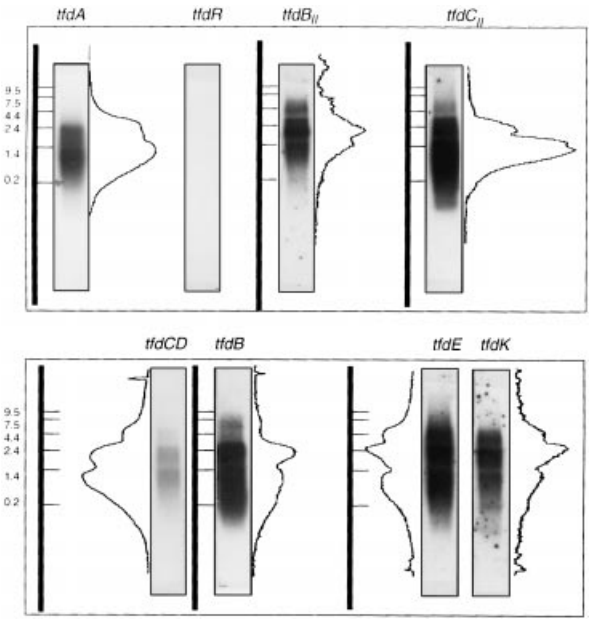

Fig. 4. Northern analysis of the different *tfd* transcripts by probing with radioactively labelled *tfdA*, *tfdR*, *tfdB$_{II}$*, *tfdC$_{II}$*, *tfdCD*, *tfdB*, *tfdE* and *tfdK* DNA fragments. Approximately 0.5 μg of total RNA was blotted in each lane. Shown are lanes with total RNAs extracted from chemostat cultures of *R. eutropha*, exposed for 15 min to the pulsed shift of 1 mM 2,4-D. No hybridization signal was found in total RNAs isolated from the culture before the pulsed shift to 1 mM 2,4-D (not shown). Size markers are indicated on the left in kilobases. Density profiles of the hybridization signals are indicated on the right.

transcripts longer than 4 kb, also including *tfdB$_{II}$* and *tfdK*. Hybridization with a *tfdB$_{II}$* or with a *tfdK* probe again revealed a relatively weak 4 kb transcript, which might be formed by a transcript from both the *tfdB$_{II}$* and the *tfdK* genes. No visible transcripts were detected on blots hybridized with a probe for *tfdR*, which is probably because of the low expression level of *tfdR*.

Transition phase of R. eutropha *cells and steady-state* tfd *expression at 0.1 M or 0.01 mM 2,4-D*

At shifts of 0.1 M and 0.01 mM 2,4-D to fructose-grown steady-state *R. eutropha* cells, only one single burst of mRNA synthesis occurred (Fig. 2). The transition phase of elevated mRNA synthesis, which lasted for about 2 h (in the case of 0.1 mM 2,4-D), was accompanied by removal of 2,4-D in the reactor to undetectably low levels (Fig. 3B). Interestingly, no decrease in 2,4-D occurred in the first 20 min after the pulsed shift, and no 2,4-DCP could be measured in the supernatant of the chemostat culture. There were no significant changes detected in the optical density (OD) of the culture during the transition phase (not shown). Activities of chlorocatechol 1,2-dioxygenase increased immediately after the pulsed shift to reach a plateau of around 25 mU mg^{-1} protein after 2 h and thereafter. The drop in *tfd* mRNA levels occurred after excess 2,4-D had disappeared from the reactor (Fig. 3B).

At a pulsed shift of 10 μM 2,4-D, the response of *R. eutropha* cells was even shorter. No 2,4-D or 2,4-DCP could be measured at such low substrate concentrations and high cell biomass. An increase in oxygen consumption was measured during the first 20 min immediately after the pulsed shift, but no significant changes in OD of the culture were detected (not shown). Activities of chlorocatechol 1,2-dioxygenase rose directly after the 2,4-D pulse, remained at a rather low level of 2–5 mU mg^{-1} protein and seemed to disappear slowly from the culture after prolonged incubation at 20 mM fructose plus 10 μM 2,4-D.

In general, mRNA levels of the *tfd* genes during steady state (i.e. when culture parameters no longer changed) were significantly higher than before 2,4-D induction. Exceptions to this were *tfdK* and *tfdC$_{II}$* mRNAs during the 10 μM shift and *tfdF* during the 0.1 mM shift (Table 1).

R. eutropha *enters a complex transition phase upon exposure to 1 mM 2,4-D*

After the addition of 2,4-D to 1 mM, degradation of 2,4-D started around 15 min after its addition at an initial rate of 1.1 mM h^{-1} (Fig. 3A). This coincided with a sharp increase in oxygen consumption (Fig. 5A) and a concomitant accumulation of 2,4-DCP in the reactor at an initial rate of ≈ 0.6 mM h^{-1}. Two minutes after the shift, the activity of chlorocatechol 1,2-dioxygenase had increased to 5 mU mg^{-1} protein (as opposed to 1 mU during growth on fructose alone). After 1 h, chlorocatechol 1,2-dioxygenase activity reached 60 mU. Gradually, both the disappearance of 2,4-D and the formation of 2,4-DCP slowed down: 2,4-D reached a minimum concentration of between 0.05 and 0.019 mM, and 2,4-DCP a maximum of 0.32–0.35 mM in the reactor (concentration range in the two repetitions of the experiments). From 2 h after the shift, 2,4-D accumulated in the reactor from the incoming medium, suggesting that it was no longer degraded. The same was true for fructose (Fig. 5A), and oxygen consumption was zero. 2,4-DCP was only disappearing from the culture by means of dilution with fresh medium. This indicated that the cells were no longer respiring actively. The cells started to wash out from the culture, as judged from OD measurements (Fig. 5A) and from agar plate counts on nutrient broth or MM plus 2,4-D (not shown). This stage of the transition phase coincided with a strong decrease in the *tfd* mRNA levels to low uninduced values (Fig. 5B). Total protein content of the cells decreased steadily, whereas total RNA per cell remained more or less constant and decreased only slightly in the final stages of this phase (not shown). Chlorocatechol

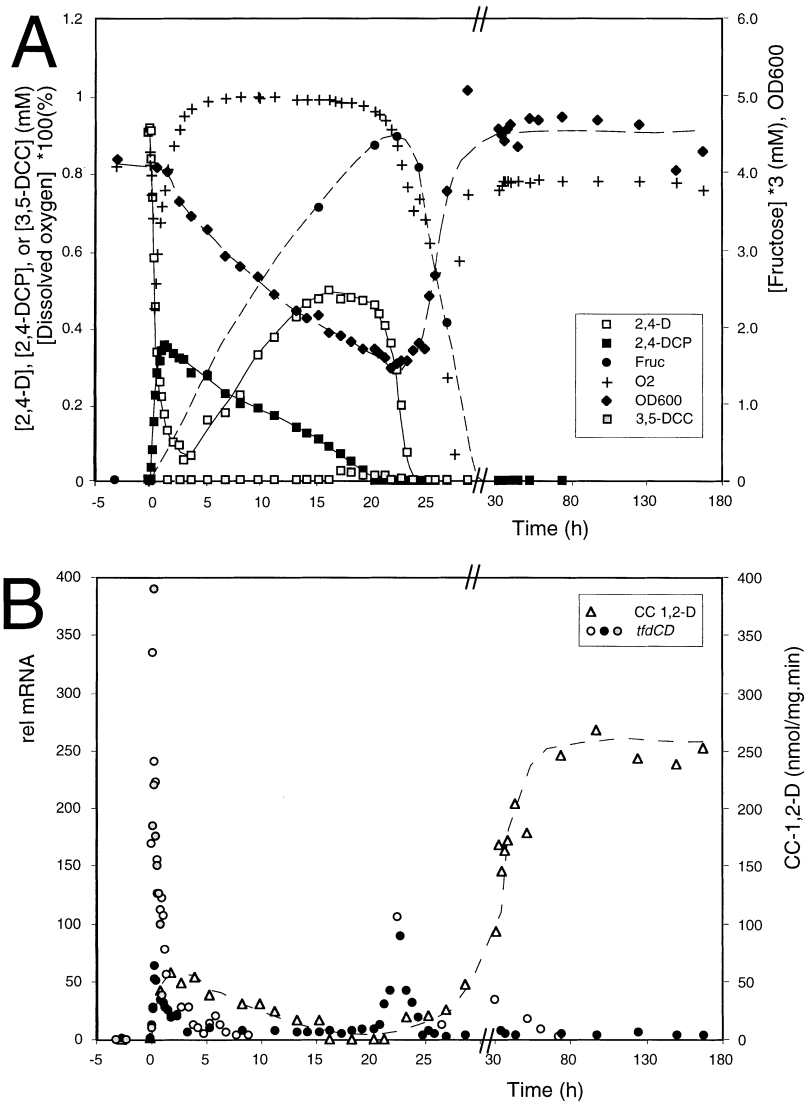

Fig. 5. Overall dynamic response of *R. eutropha* JMP134 (pJP4) cells during the transient phase of *tfd* gene induction to 1 mM 2,4-D and subsequent steady state.
A. Changes in culture parameters.
B. Relative levels of the *tfdCD* mRNA and activities of chlorocatechol 1,2-dioxygenase (CC 1,2-D). Transition changes of all *tfd* mRNAs tested, except *tfdR* and *tfdS* mRNA, behaved similarly to those shown here for *tfdCD*. The addition of 2,4-D to cells in a steady state on 20 mM fructose occurred at time zero. Note the different scales within the time axis of each experiment and the different axes for the parameters. Open, closed and grey circles for the *tfdCD* mRNA levels represent values from three independent experiments.

1,2-dioxygenase activity also gradually disappeared after 4 h to levels around 20 mU mg^{-1} protein.

By 17 h, 2,4-DCP degradation appeared to resume, with 2,4-DCP concentrations reaching undetectable levels within 3 h. During this time, there was a transient accumulation of 3,5-DCC in the reactor to a maximal concentration of 25 μM, and the 2,4-D concentration levelled off to about 0.5 mM (Fig. 5A). In the following 3.5 h, the excess 2,4-D was completely degraded, and oxygen consumption had increased. Between 20 and 25 h, we observed a second activation of all *tfd* genes (except *tfdR/S*) and another decrease to low levels (Fig. 5B). This second rise occurred shortly after the 2,4-D concentration ceased to increase and the disappearance of 2,4-DCP accelerated. The mRNA levels of the second induction reached similar or slightly lower values to those of the first induction phase, depending on the gene analysed. Once the excess 2,4-D was used and *tfd* mRNA levels were low again, consumption of fructose started, paralleled by a high rate of oxygen consumption (Fig. 5A). Complete removal of fructose took about 4 h. During this time, cell biomass more than tripled. The calculated doubling time of 2.5 h during this period was close to the fastest doubling time of 1.6 h of *R. eutropha* JMP134 (pJP4) on fructose (Müller and Babel, 1996). The protein content per cell went up again to levels similar to those before the shift, and there was a transient increase (almost twofold) in total RNA content per cell (not shown). A second induction of chlorocatechol 1,2-dioxygenase activity followed the second peak of *tfd* gene activation. This time, the activity of the enzyme continued to rise until the steady state on fructose plus 2,4-D was reached, when it levelled off to an average of 249 ± 11 mU mg^{-1} protein (Fig. 5A).

The utilization of excess fructose coincided with the second low of *tfd* gene expression (Fig. 5). However, once

this excess was depleted, there was another rise in mRNA levels (Fig. 5B). Within 5 h, the levels of mRNA now stabilized at constant values (Table 1). Among the *tfd* genes expressed highest at steady state were *tfdA*, $tfdB_{II}$ and *tfdK*, with an estimated average of 14–30 mRNA copies per cell, followed by *tfdB*, *tfdCD*, *tfdE*, *tfdF*, $tfdC_{II}$, $tfdE_{II}$ and $tfdF_{II}$ with 2–10 copies per cell (Table 1). Expression of *tfdR* (and *tfdS*) fluctuated slightly around an average value of between one and two copies per cell (Table 1). Biomass, protein content, total RNA and oxygen consumption now also remained stable. Incoming fructose and 2,4-D were consumed to concentrations below the detection limits, and the pathway intermediates 2,4-DCP and 3,5-DCC were no longer detectable. This new steady state on fructose and 2,4-D was reached after a total of 50 h.

Discussion

Study of gene expression is a powerful tool for monitoring bacterial responses to changing environmental conditions. For this purpose, the use of reporter gene constructs has been very successful. Specific responses of the bacterial cells have been analysed upon a variety of extracellular signals, substrates or stresses by measuring the activity or presence of a reporter protein after specific time intervals (Olsson *et al*., 1988; Forsberg *et al*., 1994; Sticher *et al*., 1997; Vollmer *et al*., 1997). However, the first signs of cellular response are changes in mRNA transcript levels, rather than enzyme activities. As most mRNA transcripts in bacteria have a half-life of a couple of minutes, response studies have to be performed on a detailed dynamic and temporal scale in order to measure variations in intracellular mRNA pools precisely upon changing environmental conditions. The method we used to study expression of the *tfd* pathway genes (for 2,4-D degradation) in *R. eutropha* JMP134 (pJP4) (chemostat cultivation and fast mRNA extraction plus dot-blot hybridizations) allowed the simultaneous analysis of 14 mRNA species with a resolution time of 3–5 min. Our results clearly showed fluctuations in mRNA abundances not observed previously and also revealed interesting details about *tfd* gene response intensity and time, which would certainly have been missed when analysed at a lower frequency or using reporter proteins and native enzyme activities.

Almost all of the *tfd* mRNA transcripts (except for that of *tfdR/S*) showed a common time-dependent expression: upon 2,4-D presence, a steep increase to a sharp peak and a subsequent return to lower levels, which were still higher than levels measured during uninduced conditions. Although this is the type of temporal response observed in various other systems (Baumann *et al*., 1996; Schwartz *et al*., 1998; Iyer *et al*., 1999), there is no a priori reason why this 'overshooting' should occur. One explanation could be that the mRNA synthesis rate (in this case, from the TfdR/S-regulated promoters) is directly dependent on the concentration of the intracellular signal molecule. Fluctuations in the intracellular concentration of the signal molecule itself would then modulate the responsive promoters likewise. In the 2,4-D degradation pathway, the signal for TfdR/S-mediated expression is 2,4-dichloromuconate (Filer and Harker, 1997), a pathway intermediate resulting from the cleavage of 3,5-dichlorocatechol (Fig. 1). Low constitutive enzyme levels in uninduced *R. eutropha* cells (such as the 1 mU of chlorocatechol 1,2-dioxygenase activity found during growth on fructose) would result in conversion of a small amount of 2,4-D into 2,4-dichloromuconate initially. This would trigger stronger expression of the *tfd* genes and lead to synthesis of the 2,4-D pathway enzymes and accumulation of pathway intermediates. However, as soon as the enzyme for 2,4-dichloromuconate conversion itself (i.e. chloromuconate cycloisomerase, product of *tfdD*) is present at higher levels in the cell, the concentration of 2,4-dichloromuconate drops and causes a decrease in *tfd* gene expression. The final steady-state values for mRNA transcript abundances were found to be dependent on the added 2,4-D concentration. Such a correlation was also observed for hydrogenase transcript pools and hydrogenase activities in *R. eutropha* H16 (Schwartz *et al*., 1998). At higher 2,4-D concentrations in the feed, more *tfd* mRNA was detectable during steady state of the culture. Consequently, this would be the result of the constant higher pool of 2,4-D intermediates (including 2,4-dichloromuconate) present in the cell at a higher 2,4-D flux through the metabolic pathway. When providing 10 μM 2,4-D in the feed, we observed that transcription of *tfdK* and $tfdC_{II}$ was no longer activated compared with uninduced conditions.

We found a significant correlation between the position of *tfd* genes relative to the TfdR/S binding site and the time of appearance of that part of the corresponding transcript after 2,4-D induction. The *tfdA* and *tfdC* genes that are immediately downstream of a TfdR/S binding site (Streber *et al*., 1987; Leveau and van der Meer, 1996) were expressed earliest (Fig. 3). The same was found for $tfdC_{II}$, which is preceded by $tfdD_{II}$ with respect to the TfdR/S binding site (Matrubutham and Harker, 1994). The further downstream a particular gene was located, the longer it took for its mRNA to appear (Fig. 3). This would suggest that *tfdCDEFB* and $tfdD_{II}C_{II}E_{II}F_{II}B_{II}K$, are each synthesized as single long transcripts, which was confirmed for the *tfdCDEFB* genes. The absence of a visible 7 kb transcript for the second *tfd* cluster and the much higher relative abundance of mRNAs probed with $tfdB_{II}$ or *tfdK* versus *tfdF* suggests that those genes are transcribed in two units, i.e. $tfdD_{II}C_{II}E_{II}F_{II}$ and $tfdB_{II}K$. This would imply, however, that the later appearance of the $tfdB_{II}K$ mRNAs results from a different regulatory signal or perhaps a weaker binding affinity of TfdR/S to a site

upstream of $tfdB_{II}$. The different signal intensities observed for genes close to a TfdR promoter (e.g. *tfdCD*) compared with more distal genes (e.g. *tfdF*) might be caused by differential mRNA stability or premature transcription termination, as observed for the *cbb* genes that encode CO_2 assimilation in *R. eutropha* H16 (Schaferjohann *et al*., 1996).

The specific oscillatory behaviour of *tfd* gene expression at concentrations of 1 mM 2,4-D seems to be dictated by the accumulation of 2,4-DCP and its toxic effects on cell metabolism. A bacteriostatic effect of 2,4-DCP results from dissipating the pH gradient across the cytoplasmic membrane and causing efflux of small molecules such as ATP (Heipieper *et al*., 1991). Toxicity effects of 2,4-DCP were also observed in stirred tank reactors for microbiological treatment of 2,4-D-contaminated waste waters (Oh and Cha, 1996), and in 2,4-D-amended soils (Short *et al*., 1991). Apparently, during the transition phase, 2,4-DCP was produced faster than it became degraded and became toxic at levels higher than 0.3 mM (in the reactor medium). One explanation for this accumulation is an imbalance in initial mRNA synthesis rates between *tfdA* and *tfdB* (and possibly $tfdB_{II}$), resulting in a temporary 100-fold abundance of *tfdA* mRNA over *tfdB* mRNA (Fig. 3), compared with only twofold during steady state (Table 1). This may have resulted in much higher levels of 2,4-D-dioxygenase (TfdA) than 2,4-DCP hydroxylase shortly after the pulsed shift and, consequently, accumulation of 2,4-DCP. The finding that 2,4-DCP conversion is the rate-limiting step in 2,4-D metabolism was also observed in *Burkholderia cepacia* carrying plasmid pJP4 (Daugherty and Karel, 1994). Residual TfdB(II) enzyme activity may have helped the cells in finally recovering from the 2,4-DCP intoxication. The second increase in *tfd* mRNA levels was the result of a temporarily higher level of 2,4-dichloromuconate in the cells, formed from the excess of accumulated 2,4-D in the reactor. This is also supported by the small transient increase in concentration of 3,5-dichlorocatechol (the precursor of 2,4-dichloromuconate) in the culture supernatant during the period (Fig. 5A). Interestingly, the excess 2,4-D was used up before fructose degradation resumed. Perhaps fructose utilization is repressed by 2,4-D or one of its cellular metabolites. Preferential substrate utilization is known in *R. eutropha* for benzoate repressing acetate utilization (Ampe and Lindley, 1995). The repression occurs at the level of transcription from the *acoE* gene, encoding acetyl coenzyme A (acetyl-CoA) synthetase, and is mediated not by benzoate itself, but by catechol, one of its metabolites. The second drop in *tfd* mRNA levels coincided with the disappearance of the excess 2,4-D in the reactor. Perhaps the excess fructose in the reactor in its turn exerted a temporary repressive effect on *tfd* expression, as *tfd* mRNA levels dropped below those found during the final steady state (during which the actual concentrations of fructose and 2,4-D in the reactor were practically zero). However, as the culture itself was also growing rapidly at this point, increased total RNA content may have led to an underestimation of the relative levels of specific *tfd* mRNAs.

The organization of the 2,4-D pathway genes in *R. eutropha* JMP134 (pJP4) is the outcome of a relatively recent evolutionary process (Fulthorpe *et al*., 1995; van der Meer, 1997), mediated in part by the insertion sequence ISJP4 (Leveau and van der Meer, 1997). The insertion brought in new gene functions (i.e. $tfdR$-$tfdD_{II}C_{II}E_{II}F_{II}B_{II}$ and *tfdK*), but disturbed the open reading frame of a regulatory gene *tfdT* (Leveau and van der Meer, 1997), which posed the necessity for another regulatory system to control expression of the *tfdCDEF* and *tfdB* genes. This task is performed by the *tfdR* (and *tfsS*) gene product (Leveau and van der Meer, 1996), which leads to the specific coordinated expression of the complete *tfd* pathway. The discovery that the second *tfd* gene cluster is also transcribed during exposure to 2,4-D adds yet another dimension to the complexity of this novel catabolic pathway.

Experimental procedures

Induction experiments

Ralstonia eutropha JMP134 (pJP4) was cultivated continuously at 30°C in a 2.5 l reactor (MBR). This strain is able to use 2,4-D as sole carbon and energy source (Don and Pemberton, 1981). The reactor was operated with a volume of 1.2 l at a dilution rate of 0.05 h^{-1} under carbon-limited conditions. Growth medium for uninduced conditions (feed 1) was based on *Pseudomonas* defined mineral medium (MM; Gerhardt *et al*., 1981), with a pH of 6.8, and with 20 mM fructose. Cells were grown for at least eight volume changes on feed 1 before being exposed to 2,4-D. This was achieved in a 'block' manner by adding concentrated 2,4-D solution into the reactor to reach a concentration of 0.01, 0.1, 0.5 or 1 mM instantaneously, and changing to growth medium 2, which was identical to feed 1 but supplemented with 0.01, 0.1 or 1 mM 2,4-D. Each chemostat experiment was performed at least twice independently. Throughout the entire experiment, we monitored changes in the following parameters: mRNA levels of the *tfd* genes (i.e. *tfdA*, *tfdB*, *tfdCD*, *tfdE*, *tfdF*, $tfdB_{II}$, $tfdC_{II}$, $tfdE_{II}$, $tfdF_{II}$, *tfdK*, *tfdR/S*, *tfdT*) and that of the 16S rRNA genes, activities of chlorocatechol 1,2-dioxygenase, cell biomass, concentrations of fructose, 2,4-D, 2,4-DCP and 3,5-DCC in the medium, and oxygen consumption. Biomass was followed by measuring the optical density (OD) at 600 nm. Viability of the cells was determined by counting colony-forming units (cfu) after plating diluted culture aliquots on MM agar plates supplemented with 5 mM 2,4-D or on Nutrient Broth (NB; Biolife) plates.

Analytical methods

Fructose concentrations were determined enzymatically using a sucrose/D-glucose/D-fructose kit from Boehringer

Mannheim. The limit of detection for fructose was about 0.1 mM. Concentrations of 2,4-D, 2,4-DCP and 3,5-DCC in the culture medium were determined on a Gynkotek HPLC system consisting of a Gina 50 automated injection module, an M480 G gradient pump, on-line degasser and a UVD 340S photodiode array detector. Samples were acidified and 0.2 μm or 0.45 μm filtered before analysis. The compounds were separated on a Nova-Pak C18 column (Waters-Millipore) using an eluent of 70% methanol–30% 50 mM NaH_2PO_4 (pH 3) (v/v) at a flow rate of 0.7 ml min^{-1}, and subsequently detected at a wavelength of 230 nm. The detection limit for these compounds was ≈ 5 μM.

Chlorocatechol 1,2-dioxygenase assay

Samples of 3 ml were taken from the reactor, and crude cell extracts were prepared as described elsewhere (Leveau and van der Meer, 1996). Chlorocatechol 1,2-dioxygenase activity was determined as described previously (Dorn and Knackmuss, 1978a,b) using 3-chlorocatechol as a substrate (a kind gift from B. Jakobs, GBF, Braunschweig, Germany). Protein concentrations of the cell-free extracts were determined according to the method of Bradford (1976) using BSA as a standard.

Plasmid constructs for the in vitro *synthesis of sense and antisense* tfd *probes*

From basically every gene of the *tfd* clusters, short fragments between 250 bp and 900 bp were cloned into pGEM-5Zf or pGEM-7Zf (Promega) and propagated in *Escherichia coli* DH5α (Sambrook *et al*., 1989) (Fig. 1). Sources of the *tfd* DNA were cloned fragments from the catabolic plasmid pJP4 of *R. eutropha* JMP134 (Don *et al*., 1985; Leveau and van der Meer, 1996; 1997). All clones were verified by restriction mapping and/or partial DNA sequencing.

In vitro *synthesis of sense and antisense* tfd *probes*

One microgram of each pGEM-derived plasmid (see above) was linearized by restriction enzyme digestion in such a way as to allow insert-specific transcription from either the T7 or the SP6 promoter. *In vitro* transcription reactions were carried out with biotin-16-UTP and T7 or SP6 RNA polymerase as suggested by the manufacturer (Boehringer Mannheim). After synthesis, the template DNAs were degraded by incubation with RNase-free DNase I (Boehringer Mannheim), and the RNAs were precipitated. This procedure was performed successfully for all probes, except for $tfdD_{II}$. For unknown reasons, insufficient antisense transcripts were formed from the template DNA of $tfdD_{II}$. Therefore, $tfdD_{II}$ was excluded from the analysis. All probes were checked for their specificity in Southern hybridizations with total genomic DNA of *R. eutropha*, digested with different restriction enzymes and with the original plasmids containing the cloned fragments of pJP4 with the *tfd* genes on them. In all cases, the expected banding patterns were observed. Slight cross-hybridization was found between probes for similar genes (e.g. *tfdC* probe with the $tfdC_{II}$ gene); however, these signals could only be observed upon very long exposure and contributed to less than 1% of the hybridization signals derived with the true probe target. Antisense probes for *tfdA*, *tfdCD* and *tfdB* were also checked in Northern hybridizations with total RNA isolated from *R. eutropha* during steady-state growth on fructose and during growth on fructose plus 2,4-D (see below). For the *tfdCD* antisense probe, a small cross-hybridization with the ribosomal RNAs was detected, which may have resulted in overestimating the background uninduced level of transcription from the *tfdCD* genes.

RNA extraction

Samples (3–6 ml) were taken directly from the chemostat. Cells spun down in a 30 s centrifugation step, and the pellets were immediately resuspended in saline TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl), buffer II (20 mM sodium acetate, 1 mM EDTA, 0.5% SDS, pH 5.5) and acid phenol at 60°C as described by Aiba *et al*. (1981). Total RNAs were purified further by phenol–chloroform extractions, precipitation and DNase I digestion (RNase-free). Finally, RNAs were stored with two volumes of ethanol and 0.1 volume of sodium acetate (pH 5.2) at −20°C. Volumetric losses during extraction equalled 40% of the initial sample volume. RNA concentrations were determined spectrophotometrically.

Northern hybridizations of tfd *mRNAs*

Total RNAs (0.1, 1 and 10 μg) from uninduced cells (i.e. during growth on 20 mM fructose) and from induced cells (i.e. 10–20 min after the addition of 2,4-D) were treated with glyoxylate to denature and then separated on phosphate-buffered agarose gel as described by Ausubel *et al*. (1995). An RNA size marker (Life Technologies) was included on each side of the gel. After electrophoresis, the gels were blotted onto Hybond N-Plus membrane (Amersham) by capillary action. Membranes were treated with UV light in a Stratalinker to fix RNAs irreversibly. Hybridization was carried out with radioactive-labelled probes at 68°C. As probes, we used DNA inserts of the various pCBA plasmids, recovered by appropriate restriction enzyme digestion and labelled using a random-primed DNA labelling kit (Boehringer Mannheim).

Dot-blot hybridization of tfd *mRNAs*

Total RNAs were recovered by centrifugation and washing with 70% ethanol, and dissolved in 350 μl of water. Tenfold (for all time samples) and 100-fold (for samples in peaks) dilutions of the RNAs were prepared. Equal volumes (25 μl) of total RNA for each time sample and for each dilution were blotted onto positively charged nylon membranes (Qiagen) in a dot-blot manifold (Gibco Life Technologies) containing a 96-well 3 mm gasket. Multiple identical blots were prepared directly after each other, as each blot was only used for a single hybridization. Included on each blot were a series of DNA standards. These standard series were composed of dilutions of the plasmid DNA containing the insert used for producing the complementary antisense RNA and of aliquoted dilutions of total genomic DNA of *R. eutropha* JMP134 (pJP4). Concentrations of plasmid and total DNA were determined from comparing intensities of serially diluted samples, stained

with ethidium bromide and irradiated with UV light, with those of a λ-DNA standard (370 ng μl^{-1}; Appligene).

Prehybridization, hybridization of the membranes to the antisense biotin-labelled mRNAs and streptavidin–alkaline phosphatase detection of the bound probes was carried out as described previously (Baumann *et al*., 1996). Membranes were exposed for different time periods to Amersham Hyperfilm MP. Other RNAs isolated from the same chemostats at regular time intervals were dot-blotted separately and used for hybridizations with sense probes.

The only exception to this protocol was hybridizations with a probe for the 16S rRNA. This probe consisted of an oligonucleotide (EUB338, 5′-GCTGCCTCCCGTAGGAGT-3′) (Amann *et al*., 1995) that was labelled with biotin-16-ddUTP using terminal transferase according to the instructions of the supplier (Amersham). Hybridization temperature and conditions for the EUB probe were 2 h at 55°C, after which the streptavidin–alkaline phosphatase detection was carried out.

Quantification of the mRNA levels

Autoradiograms were scanned on a laser densitometer (Molecular Dynamics). The density volumes of the dots were quantified with IMAGEQUANT (Molecular Dynamics) by applying a grid on the scanned blot and using the function 'local background' for background correction. Quantification data were then processed further with EXCEL (Microsoft) and KALEIDAGRAPH PPC (Synergy Software). mRNA levels were calculated relative to the densities of the DNA standard series and to the amount of total RNA blotted, as outlined below. A non-linear standard curve was obtained by expressing all DNA standards as total amount of *tfd* gene copies per dot versus the observed density volume. For this, we assumed a size of the *R. eutropha* chromosome of 3.5 Mb and a size of 3.5 kb for the pCBA plasmids and, hence, molecular masses of 3.72×10^{-6} μg and 3.72×10^{-9} μg respectively. As we found previously that pJP4 occurs in monocopy in *R. eutropha* (Leveau and van der Meer, 1997), this implied that 1 μg of *R. eutropha* JMP134 (pJP4) DNA contained 2.7×10^{8} *tfd* gene copies (and 5.4×10^{8} for *tfdR* because of its duplication). Parameters of the non-linear standard curves were derived with KALEIDAGRAPH using the general hyperbolic equation $y = m1*m0/(m2 + m0)$, where m0 stands for the amount of copies per dot, y = the density volume of the dot, and m1 and m2 are the curve parameters. Parameters that gave the best fit were used to calculate total amounts of *tfd* mRNA copies in each time sample. For peak values (i.e. with the highest density volumes), we used the 100-fold diluted RNAs wherever possible; otherwise, we used the density volumes from the 10-fold diluted RNAs. Average differences between amounts of mRNA copies calculated from undiluted samples versus 10-fold diluted ones was a factor of 9; between 10-fold and 100-fold diluted RNAs a factor of 4. Finally, the amounts of mRNA copies were divided by the total RNA amount blotted (per μg) and by a factor of 10^{8}, assuming that ≈ 1 μg of RNA was isolated from 10^{8} cells (including losses in our sampling procedure). These final values are referred to as relative mRNA levels and can be interpreted as the average mRNA copy number per cell. Aspecific background hybridization to DNA contamination in isolated total RNA was low: hybridization with sense *tfd* probes gave, on average, relative mRNA levels between 0 and 0.03 throughout the experiment (not shown), which were well below the antisense values.

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