

# Transcriptional control of lipid metabolism by the MarR-like regulator FamR and the global regulator GlxR in the lipophilic axilla isolate *Corynebacterium jeikeium* K411

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## Summary

**Corynebacterial fatty acid metabolism has been associated with human body odour, and is therefore discussed as a potential target for the development of new deodorant additives. For this reason, the transcription levels of *fad* genes associated with lipid metabolism in the axilla isolate *Corynebacterium jeikeium* were analysed during growth on different lipid sources. The transcription of several *fad* genes was induced two- to ninefold in the presence of Tween 60, including the acyl-CoA dehydrogenase gene *fadE6*. DNA affinity chromatography identified the MarR-like protein FamR as candidate regulator of *fadE6*. DNA band shift assays and *in vivo* reporter gene fusions confirmed the direct interaction of FamR with the mapped *fadE6* promoter region. Moreover, DNA affinity chromatography and DNA band shift assays detected the binding of GlxR to the promoter regions of *fadE6* and *famR*, revealing a hierarchical control of *fadE6* transcription by a feed-forward loop. Binding of GlxR and FamR to additional *fad* gene regions was demonstrated *in vitro* by DNA band shift assays, resulting in the co-regulation of *fadA*, *fadD*, *fadE* and *fadH* genes. These results shed first light on the hierarchical transcriptional control of lipid metabolism in *C. jeikeium*, a pathway associated with the development of human axillary odour.**

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## Introduction

The skin dweller *Corynebacterium jeikeium* is an opportunistic human pathogen, which is frequently recognized in medical facilities causing a variety of infections including sepsis, skin lesions, and endocarditis (van der Lelie *et al.*, 1995; Mookadam *et al.*, 2006). Especially immunocompromised patients carrying prosthetic valves or catheters are affected and the elimination of *C. jeikeium* is often complicated by its multi-resistance against commonly used antibiotics (Olson *et al.*, 2009; Ifantidou *et al.*, 2010). However, *C. jeikeium* is part of the skin flora of healthy individuals and predominantly colonizes moist areas of the human body, like the axilla. Here, *C. jeikeium* and other aerobic corynebacteria contribute to the generation of volatile compounds of axillary odour (James *et al.*, 2004; Natsch *et al.*, 2006) by metabolizing certain precursor molecules in human sweat secretions, like glutamine-conjugated short-chain fatty acids (Natsch *et al.*, 2003). The medical as well as the cosmetic importance are consequences of a lipid-dependent lifestyle, because *C. jeikeium* can feed on serum fatty acids during infection (Brinster *et al.*, 2009) and is able to utilize fatty acids from the skin surface to produce odorous short-chain fatty acids (James *et al.*, 2004). Nowadays, the cosmetic industry is interested in the development of new deodorant additives that specifically target the metabolism of malodour causing bacteria instead of generally affecting the beneficial skin microflora (Brune *et al.*, 2006). Especially the inhibition of certain enzymatic functions that cause axillary odour and the application of fragrance precursors as alternative substrates for bacterial enzymes are currently considered (Natsch *et al.*, 2005; 2007). Therefore, the investigation of lipid metabolism and its regulation in the lipophilic model organism *C. jeikeium* is of high relevance for the cosmetic industry.

Generally, *C. jeikeium* is unable to use sugar compounds as sole carbon source (Tauch *et al.*, 2005) and predominantly feeds on skin secretions containing a variety of different lipid compounds (Nicolaidis, 1974; Pappas, 2009). Additionally unable to synthesize fatty acids *de novo*, *C. jeikeium* is strictly dependent on exogenous lipid sources in order to maintain its cell envelope and to guarantee constant energy supply (Tauch *et al.*,

2005). To compensate for its incapability in synthesizing fatty acids, *C. jeikeium* harbours a comprehensive set of enzymes involved in lipid metabolism encoded by multiple paralogous genes (Tauch *et al.*, 2005; Barzantny *et al.*, 2012a). This feature probably enables the organism to metabolize a broader range of lipid compounds, since the overall sequence identity among the paralogues is quite low, possibly counting for different enzymatic substrate specificities. The access to structurally diverse fatty acids on the skin surface and the inability of fatty acid-catabolizing corynebacteria to fully degrade skin lipids leads to the generation of volatile odour compounds. For the development of new active deodorant additives it is necessary to identify key player enzymes in this process of malodour generation to specifically target and influence enzymatic processes in the human axilla (James *et al.*, 2004).

Recently, the involvement of the global cAMP receptor protein-like corynebacterial regulator GlxR in the regulation of lipid metabolism was proposed, demonstrating the central role of this metabolic pathway in the lifestyle of *C. jeikeium* (Barzantny *et al.*, 2012b). Additionally, the putative MarR-like transcriptional regulator Jk0257 was identified in the transcriptional regulatory network (TRN) of *C. jeikeium*, which is uniquely encoded in genomes of corynebacteria residing in lipid-rich habitats (Barzantny *et al.*, 2012b). Up to now, no MarR-like protein has been associated with the regulation of lipid metabolism. Unlike *Escherichia coli*, *C. jeikeium* does not encode a FadR homologue, responsible of regulating transport, activation and  $\beta$ -oxidation of medium- and long-chain fatty acids under aerobic conditions (Feng and Cronan, 2010). In this study, we demonstrate for the first time the involvement of the MarR-like transcriptional regulator Jk0257 in the regulation of fatty acid metabolism genes and present the influence of lipid compounds on their expression. Furthermore, we highlight the role of the global corynebacterial regulator GlxR in the regulation of lipid metabolism and provide new insights into the TRN of the lipophilic axilla isolate *C. jeikeium* K411.

## Results

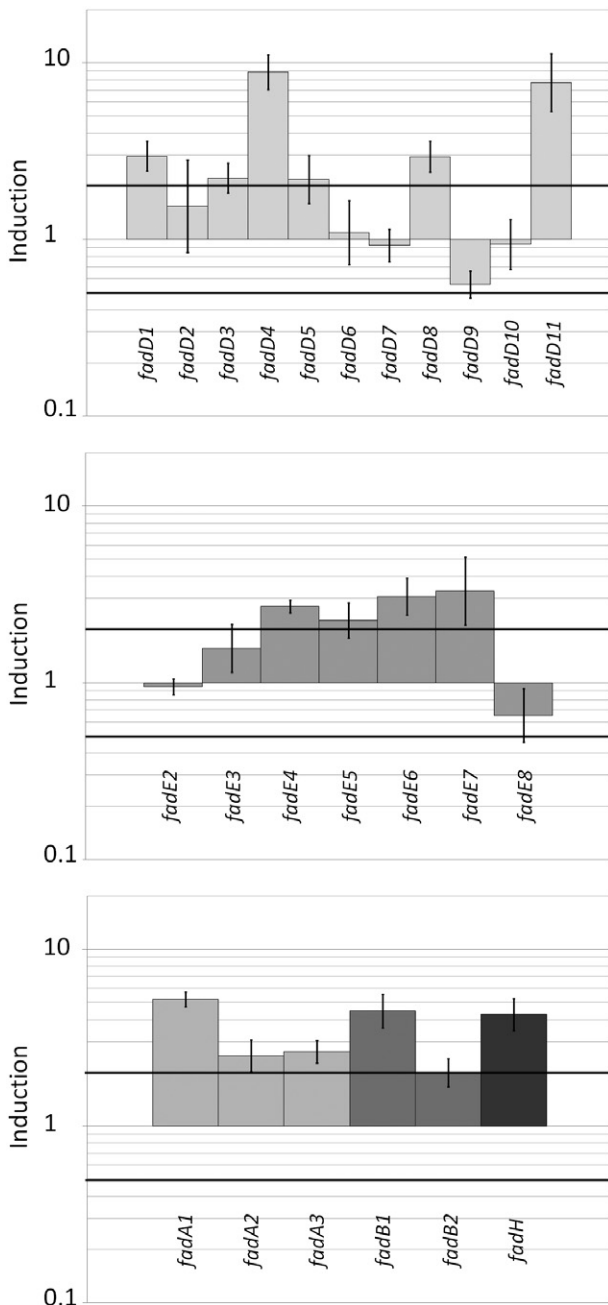
### *Differential transcription of fatty acid metabolism genes in C. jeikeium*

The *C. jeikeium* K411 genome encodes multiple paralogous genes for enzymatic functions involved in the conversion of lipid compounds (Tauch *et al.*, 2005; Barzantny *et al.*, 2012a). To investigate whether certain genes are differentially transcribed during growth on different lipid sources, the lipid-auxotrophic strain *C. jeikeium* K411 was cultivated in CJK minimal medium supplemented with either 1% (v/v) Tween 80 or Tween 60. These non-ionic surfactants are composed of a polyoxyethylenated sorbi-

tan backbone esterified to fatty acids. Tween 60 mainly contains saturated C16 and C18 fatty acids, whereas Tween 80 is a mixture of saturated C12 to C18 and unsaturated fatty acids, with oleic acid being the main component (71.3%) (Wynn and Ratledge, 2000; Vu Dang *et al.*, 2006). Subsequently, total RNA was isolated from exponentially growing cultures and real-time reverse transcriptase PCR was performed, resulting in the detection of differentially expressed fatty acid metabolism genes (Fig. 1). The transcription of several genes is induced in the range of two- to ninefold during growth on Tween 60 in comparison to growth on Tween 80 (Fig. 1). Differentially expressed genes encode acyl-CoA synthetases (FadD1/4/8/11), acyl-CoA dehydrogenases (FadE4/6/7), ketoacyl-CoA thiolases (FadA1/3), the hydroxyacyl-CoA dehydrogenase FadB1, and the 2,4-dienoyl-CoA reductase FadH. In total, we detected either the induction of gene expression by Tween 60 (11 genes), no influence on gene expression (13 genes) or no expression at all (*fadE1*). This analysis furthermore shows that all paralogues, with the exception of *fadE1*, are actively transcribed in *C. jeikeium* during growth on Tween 60 and Tween 80. However, the addition of Tween 60 clearly affects gene transcription positively and demonstrates that external lipid compounds are signals for the differential transcription of fatty acid metabolism genes. Furthermore, the results give rise to the question how the external lipid composition triggers the altered transcription levels and which transcriptional regulators are involved in the control of fatty acid metabolism genes in *C. jeikeium*.

### *Detection of proteins binding to the upstream region of fadE6*

For the detection of transcriptional regulators controlling the expression of fatty acid metabolism genes, we selected the *fadE6* gene encoding an acyl-CoA dehydrogenase (Barzantny *et al.*, 2012a). A 247 bp DNA fragment including the 5' region of *fadE6* was generated by PCR with the primer pair *fadE6*-fish-a/b (Table S1), immobilized at streptavidin-coated magnetic beads, and incubated with a combined protein extract from *C. jeikeium* cells grown in the presence of Tween 80 (1.1%), Tween 80/60 (0.1/1%) or Tween 80/40 (0.1/1%). DNA-binding proteins were visualized by SDS-PAGE and discrete protein bands were analysed by MALDI-TOF/TOF mass spectrometry and peptide mass fingerprinting. Twelve proteins were identified with significant Mascot Scores, whereof one was annotated as putative transcriptional regulator Jk0257 belonging to the MarR family of regulatory proteins (Fig. 2). Strikingly, the regulator is only encoded in sequenced genomes of the clusters 3 and 4 of the genus *Corynebacterium*, comprising many species that are characterized by a lipid-dependent lifestyle or reside in



**Fig. 1.** Differential transcription of fatty acid metabolism genes in *C. jeikeium*. Real-time RT-PCR was performed with total RNA isolated from cells grown in CJK minimal medium supplemented with 1% of Tween 80 or Tween 60. Transcription levels of *fad* genes in cells grown in the presence of Tween 60 are displayed in comparison to values detected in cells grown on Tween 80 medium. The experiment combines the results of two biological and two technical replicates. Black lines indicate the thresholds of significance (twofold induction and 0.5-fold repression). Genes investigated are: *fadD1-11*, acyl-CoA synthetase; *fadE2-8*, acyl-CoA dehydrogenase; *fadA1-3*, keto-acyl thiolase; *fadB1-2*, hydroxyacyl-CoA dehydrogenase; *fadH*, 2,4-dienoyl-CoA reductase. No mRNA was detected in case of the *fadE1* gene.

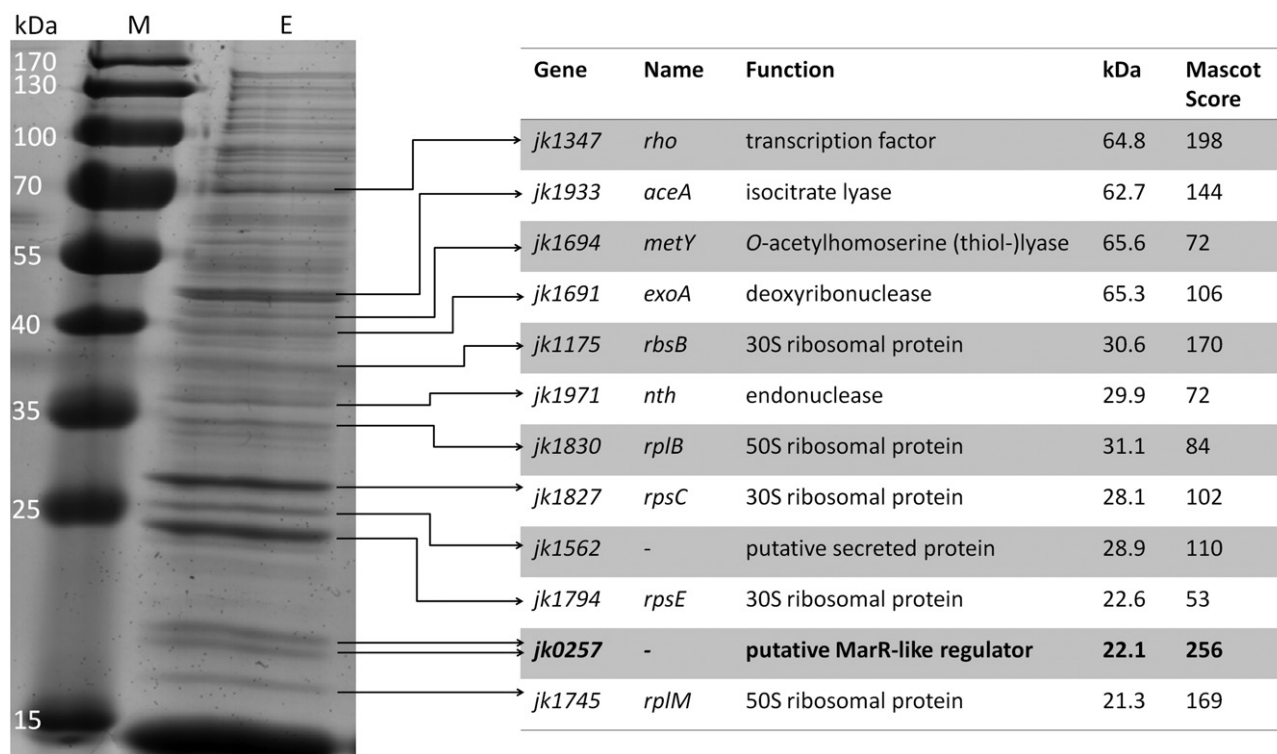
lipid-rich habitats (Khamis *et al.*, 2004; Barzantny *et al.*, 2012b). Generally, MarR-like regulators control diverse cellular processes, where they function as either activator or repressor (Perera and Grove, 2010). For the recognition and binding of specific DNA sequences, MarR homologues contain a winged helix–turn–helix motif and their binding activity can be modulated by lipophilic anionic ligands, leading to an attenuated binding to their specific DNA recognition site (Wilkinson and Grove, 2006).

#### *In vitro* binding of Jk0257 to the upstream region of *fadE6*

To verify the interaction of the MarR-like regulator Jk0257 with the upstream region of the *fadE6* gene, the regulator was heterologously expressed in *E. coli* 2566 and purified by means of an Intein-tag with the IMPACT system (Chong *et al.*, 1998). The Intein-tag was cleaved off by induced autocleavage to obtain the native Jk0257 protein for subsequent DNA band shift assays. Electrophoretic mobility shift assays (EMSA) were conducted with the purified regulator and fluorescently labelled PCR fragments of different lengths, covering 765 bp of the *fadE6* upstream region (Fig. 3A). The assays were separated in agarose gels and DNA bands were visualized by fluorescence imaging, showing that Jk0257 is able to bind to the DNA fragments 2 and 3 *in vitro* (Fig. 3A). In contrast, no binding of Jk0257 was observed with the farthest DNA fragment 1 (Fig. 3A).

Subsequently, fragment 3 was shortened to locate more precisely DNA elements necessary for the binding of Jk0257 to the upstream region of *fadE6* (Fig. 3B). The respective EMSA show a clear binding of Jk0257 to the DNA fragments 3.1 and 3.2, which is reduced with the DNA fragment 3.3 and finally lost with the DNA fragment 3.4 (Fig. 3B). Consequently, these results restrict a potential binding site for Jk0257 to a 63 nt stretch in DNA fragment 3.

The localization of DNA elements necessary for Jk0257 binding in relation to the *fadE6* promoter region is a necessary step in elucidating the regulatory mode of action of Jk0257. Generally, the interaction of regulators with DNA sequences close to the promoter region interferes with RNA polymerase recruitment, transcription initiation or mRNA elongation (Payankulam *et al.*, 2010) and therefore result in genetic repression. Otherwise, binding of DNA elements upstream of the promoter region possibly facilitates RNA polymerase binding and leads to the activation of gene expression. To further elucidate the control mechanism of *fadE6* transcription by Jk0257, the 5' end of *fadE6* mRNA was identified by 5' RACE-PCR to locate the promoter region of this gene. The transcription of the acyl-CoA dehydrogenase gene *fadE6* starts at the adenosine residue located 165 bp upstream of the ATG start



**Fig 2.** SDS-PAGE of *C. jeikeium* K411 proteins enriched by DNA affinity chromatography. A 247 bp DNA fragment covering the 5' region of *fadE6* was used for the detection of DNA-binding proteins. A combined protein extract of cultures grown on different types of Tween was incubated with the DNA-coated magnetic beads. The arrows indicate protein bands that were excised from the gel and identified by MALDI-TOF/TOF mass spectrometry. Data of identified proteins are summarized in tabular form. M, Page Ruler Prestained Protein Ladder (Thermo Scientific); E, eluted protein fraction.

codon. The deduced promoter is characterized by the -10 region TATTCT and the -35 sequence GTGCTT, with a spacer of 17 bases (Fig. 3B). Consequently, Jk0257 is able to bind DNA elements located upstream (fragment 2) and downstream (fragment 3.2) of the deduced promoter, the latter rather suggesting a repression of *fadE6* transcription by Jk0257.

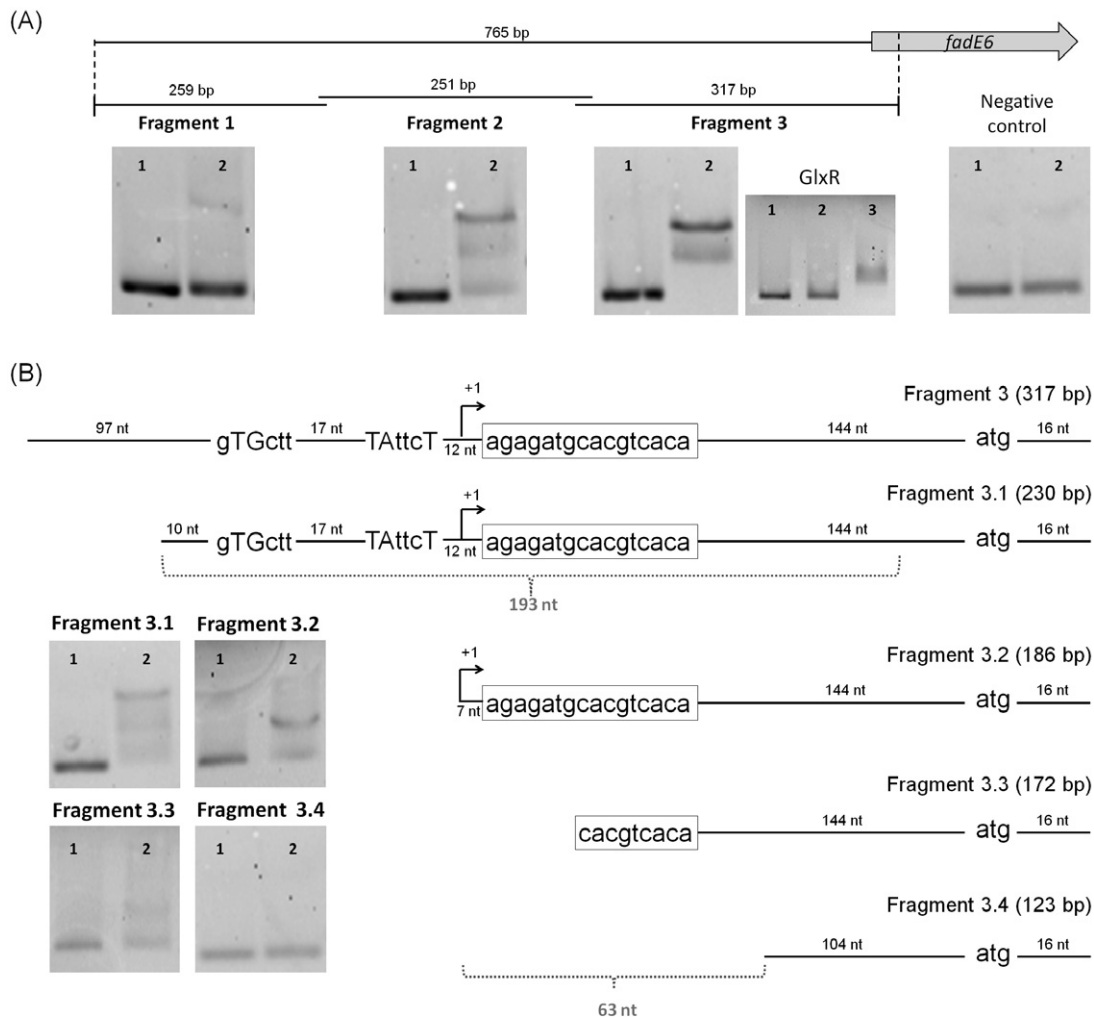
#### *In vivo* binding of Jk0257 to the upstream region of *fadE6*

To further support the observation that Jk0257 controls the transcription of *fadE6*, the promoter region of the acyl-CoA dehydrogenase gene was coupled with the reporter gene *gfp* (green fluorescent protein) and expression of the reporter protein was measured *in vivo* in the heterologous host *C. glutamicum*. This heterologous approach was selected due to the lack of an established host-vector and transformation system for *C. jeikeium*. To test the promoter activity of *fadE6* in the absence and presence of the transcriptional regulator Jk0257, a 193 bp DNA fragment covering the detected promoter region of *fadE6* (Fig. 3B) was amplified by PCR and cloned in front of the promoterless *gfp* gene of plasmid

pEPR1 (Table S1). Measurements of Gfp fluorescence in *C. glutamicum* ATCC 13032 [pEPR1-*fadE6*] showed a threefold enhanced Gfp expression (15 142 fluorescence units) when compared with the control strain *C. glutamicum* ATCC 13032 [pEPR1] (4942 fluorescence units). This result indicates that the promoter of *fadE6* is active in the heterologous host *C. glutamicum*. Furthermore, the regulatory gene *jk0257* and its native upstream region were amplified by PCR and cloned divergently onto the plasmid pEPR1-*fadE6*, resulting in the vector pEPR1-*fadE6*-*jk0257* (Table S1). A significant decrease of Gfp fluorescence back to the detected background level in *C. glutamicum* ATCC 13032 [pEPR1] was measured with the reporter construct additionally containing the *jk0257* gene (6391 fluorescence units). This experiment reveals the direct repressive interaction of the transcriptional regulator Jk0257 with the *fadE6* promoter region *in vivo*.

#### Co-regulation of *fadE6* by the global transcriptional regulator GlxR

Previous reconstruction of the TRN of *C. jeikeium* K411 led to the detection of a potential GlxR binding site in the upstream region of the *fadE6* gene (Barzantny *et al.*,



**Fig. 3.** *In vitro* binding of Jk0257 to the upstream region of *fadE6*.

A. A 765 bp fragment of the *fadE6* upstream region was subdivided into three overlapping DNA fragments. DNA band shift assays were carried out with 30 pmol of the purified Jk0257 protein and the fluorescently labelled DNA fragments 1–3. A DNA fragment containing the McbR binding site of the *cysI* gene served as a negative control. Lanes 1, labelled DNA fragments; lanes 2, labelled DNA fragments and purified Jk0257. Additionally, DNA fragment 3 was shifted with purified GlxR protein. Lane 1, labelled DNA fragment; lane 2, labelled DNA fragment and purified GlxR; lane 3, labelled DNA fragment, purified GlxR, and 0.4 mM cAMP.

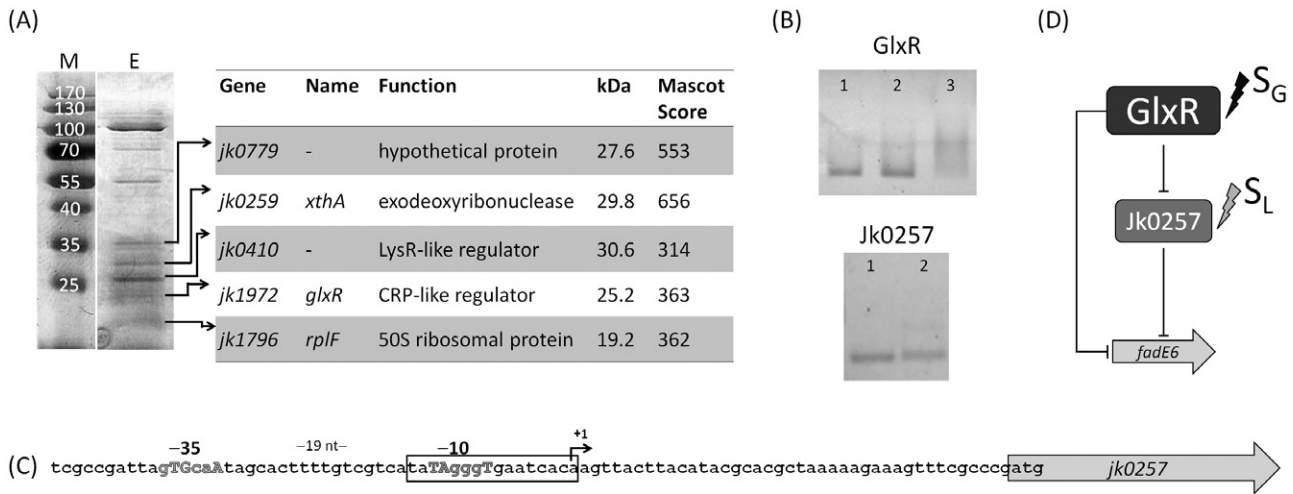
B. DNA band shift assays with shortened versions of fragment 3. The deduced promoter region of *fadE6* is displayed. The –10 and –35 hexamers are shown, in which capital letters indicate conserved nucleotides. The transcription start site (+1) of *fadE6* is highlighted by an arrow. The predicted DNA binding site of GlxR is marked with a box. The 193 bp fragment used for the *in vivo* Gfp reporter assay is marked with a brace.

2012b). However, GlxR was not enriched by previous DNA affinity experiments (Fig. 2). In order to validate the co-regulation of *fadE6* by Jk0257 and GlxR, the interaction of the GlxR protein with its predicted binding site was investigated. For this purpose, the *glxR* gene was amplified with the primer pair *glxR*-NdeI-a/*glxR*-SapI-b and cloned into the expression vector pTXB1 (Table S1). GlxR was heterologously expressed in *E. coli* 2566 and purified by means of an Intein-tag with the IMPACT system (Chong *et al.*, 1998). The purified protein was used for DNA band shift assays with fragment 3 comprising the promoter element of *fadE6* and the predicted GlxR binding site (Fig. 3B). This assay demonstrated the

binding of GlxR with the effector molecule cAMP to the upstream of *fadE6* (Fig. 3A). The location of the GlxR binding site downstream of the designated promoter region (Fig. 3B) suggests a repressive control of *fadE6* expression by GlxR.

#### *Hierarchical regulation of fadE6 by Jk0257 and GlxR*

The detected binding of the regulatory proteins GlxR and Jk0257 to the *fadE6* upstream region proposes the presence of global and local control mechanisms for *fadE6* transcription. Furthermore, the prediction of a GlxR binding site in the *jk0257* upstream region (Barzantny



**Fig. 4.** Analyses of regulatory processes controlling the transcription of *jk0257*.

A. A fragment covering the 5' region of *jk0257* was used for the detection of DNA-binding proteins. Eluted proteins were separated by SDS-PAGE. Arrows indicate protein bands that were excised from the gel and identified by MALDI-TOF/TOF mass spectrometry. Data of identified proteins are summarized in tabular form. M, Page Ruler Prestained Protein Ladder (Thermo Scientific); E, eluted protein fraction.

B. EMSA conducted with a fluorescently labelled PCR fragment covering the *jk0257* upstream region and purified regulatory proteins (GlxR or Jk0257). The assays were separated on 3% agarose gels. Lanes 1, labelled PCR product; lanes 2, PCR product and regulatory protein; lane 3, PCR product, purified GlxR, and 0.4 mM cAMP.

C. Genetic features of the *jk0257* upstream region. The transcription start site (+1) was identified by 5' RACE-PCR. The deduced *jk0257* promoter region is highlighted by bold grey letters, of which capital letters indicate conserved bases. The predicted GlxR binding site is marked with a box.

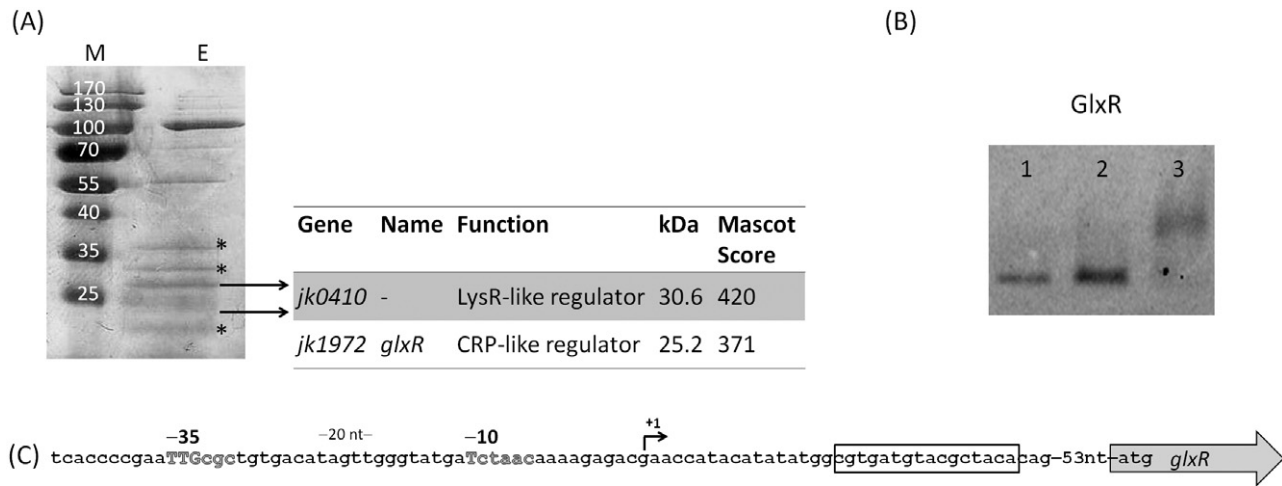
D. Regulatory network motif for the control of *fadE6* transcription. The motif of the incoherent type 2 allows the coordination of global ( $S_G$ ) and local signals ( $S_L$ ). GlxR senses the concentration of its effector molecule cAMP ( $S_G$ ) in the bacterial cell and represses *fadE6* and *jk0257* transcription accordingly. The retraction of global control by GlxR enables the local regulator Jk0257 to react to further specific signals ( $S_L$ ) and adjust *fadE6* transcription.

*et al.*, 2012b) suggests the co-regulation of *fadE6* by a hierarchical network structure. To verify the binding of the global regulator GlxR to the *jk0257* upstream region, a DNA affinity chromatography experiment was carried out. For this purpose the 5' region of *jk0257* was amplified with the biotinylated primer pair *jk0257*-fish-a/b (Table S1), immobilized at streptavidin-coated magnetic beads, and incubated with a protein extract from *C. jeikeium* cells grown in BYT medium. Subsequently, DNA-binding proteins were eluted, visualized by SDS-PAGE and identified by MALDI-TOF/TOF analysis (Fig. 4A). The binding of GlxR to the upstream region of *jk0257* was confirmed by the identification of GlxR in one of the analysed protein bands. Additionally, another DNA-binding protein was detected, annotated as the putative LysR-like regulator Jk0410. It is not clear if this regulator is somehow involved in the control of *jk0257*, because there is no information currently available for this transcription factor in *C. jeikeium*.

To further support the control of *jk0257* expression by GlxR, EMSA were carried out with the purified GlxR protein and the *jk0257* upstream region (Fig. 4B). This assay demonstrates the direct interaction of GlxR with the 5' region *jk0257* in the presence of the effector molecule cAMP. In order to predict a negative or positive regulation

of *jk0257* by GlxR, the 5' end of the mRNA was identified by 5' RACE-PCR. The transcription start of *jk0257* is located 41 bp upstream of the ATG start codon (Fig. 4C). Further upstream the -10 (TAGGGT) and -35 (GTGCAA) regions were identified with a spacer sequence of 19 nt. Consequently, the location of the GlxR binding site within the *jk0257* promoter region rather implies the repression of *jk0257* transcription by GlxR. Furthermore, a DNA band shift assay was carried out with the upstream region of *jk0257* and the encoded regulator itself (Fig. 4B). No interaction of the regulator and the 5' region of *jk0257* was detectable. Hence, an autoregulation mechanism for the *jk0257* gene is unlikely.

In summary, the regulation of *fadE6* by GlxR and Jk0257 is accomplished by a feed-forward loop of the incoherent type 2 (Fig. 4D), possibly allowing the conjunction of global and local signals for the expression of the *fadE6* gene (Mangan and Alon, 2003). This means that GlxR senses the energetic state of the bacterial cell ( $S_G$ ) and shuts down the expression of the *fadE6* and *jk0257* genes at high levels of intracellular cAMP. With decreasing cAMP concentrations, GlxR supposedly dissociates from both genetic loci thereby retracting its global control and enabling the regulator Jk0257 to 'decide' about *fadE6* expression according to local signals ( $S_L$ ).



**Fig. 5.** Analyses of regulatory processes controlling the transcription of *glxR*.

A. A DNA fragment covering the 5' region of *glxR* was used for the detection of DNA-binding proteins. Eluted proteins were separated by SDS-PAGE. Arrows indicate protein bands that were excised from the gel and identified by MALDI-TOF/TOF mass spectrometry. Asterisks mark protein bands with no significant Mascot Score. Data of identified proteins are summarized in tabular form. M, Page Ruler Prestained Protein Ladder (Thermo Scientific); E, eluted protein fraction.

B. EMSA conducted with the purified regulatory GlxR protein and a fluorescently labelled PCR fragment covering the *glxR* upstream region. The assay was separated on a 3% agarose gel. Lane 1, labelled PCR product; lane 2, PCR product and GlxR; lane 3, PCR product, GlxR and 0.6 mM cAMP.

C. Genetic features of the *glxR* upstream region. The transcription start site (+1) was deduced from 5' RACE-PCR. The *glxR* promoter region is highlighted by bold grey letters, of which capital letters indicate conserved bases. The predicted GlxR binding site is marked with a box.

#### Autoregulation of *glxR* transcription

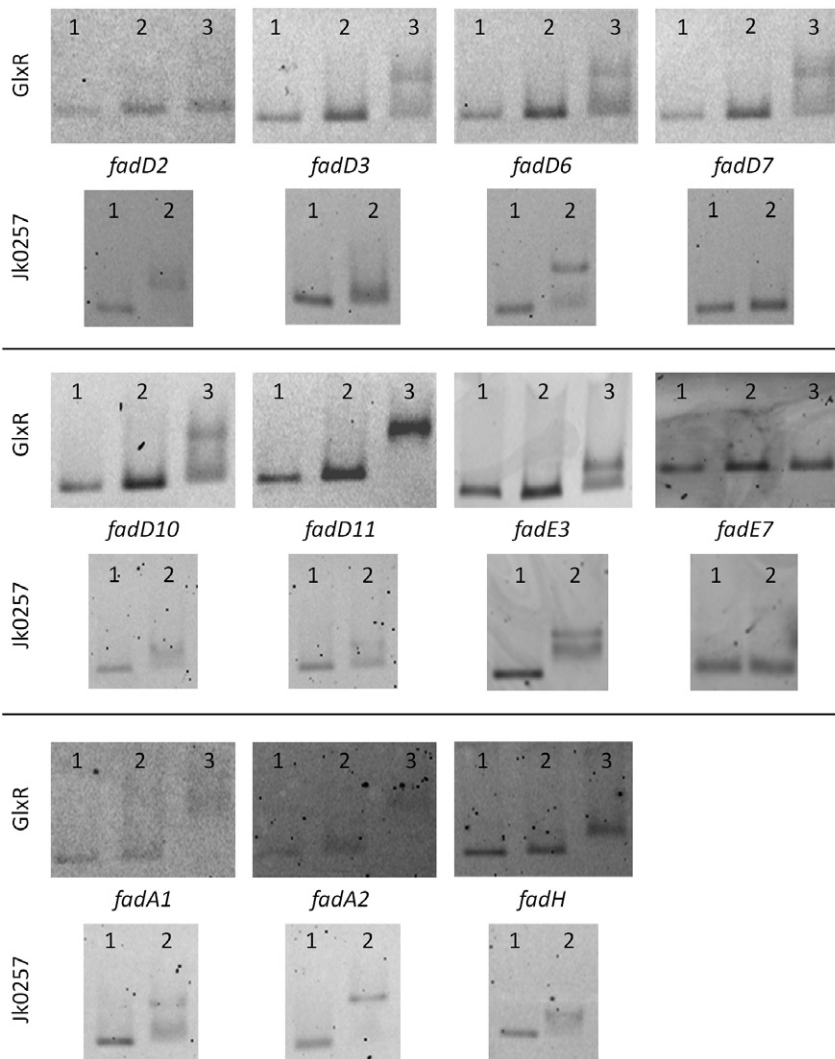
To further elucidate regulatory control mechanisms of *glxR* transcription in *C. jeikeium*, additional DNA affinity chromatography experiments were conducted with the upstream region of the *glxR* gene. Previous bioinformatic analyses identified a putative GlxR binding site in the 5' region of *glxR* (Barzantny *et al.*, 2012b). A DNA fragment covering the *glxR* upstream region with its predicted GlxR binding site was generated by PCR, immobilized at streptavidin-coated magnetic beads, and exposed to a protein extract derived from *C. jeikeium* cells grown in BYT medium. DNA-binding proteins were visualized by SDS-PAGE and analysed by MALDI-TOF/TOF mass spectrometry, leading to the detection of GlxR and the putative LysR-like transcriptional regulator Jk0410 (Fig. 5A). To verify an autoregulatory role of GlxR, DNA band shift assays were carried out with the purified regulator and a 149 bp DNA fragment comprising the upstream region of *glxR* (Fig. 5B). This assay demonstrates that GlxR is able to build a complex with the regulatory region of *glxR* in the presence of the effector substance cAMP. These data confirm the proposed autoregulation mechanism of *glxR* transcription in *C. jeikeium*, which was previously demonstrated for the amino acid producer *C. glutamicum* (Jungwirth *et al.*, 2008).

Furthermore, results of 5' RACE-PCR experiments allowed the identification of the -10 and -35 promoter elements upstream of the *glxR* coding region (Fig. 5C).

According to these results, the GlxR binding site is located downstream of the deduced transcriptional start site of the *glxR* gene. Hence, *glxR* transcription is most likely controlled by a negative autoregulation mechanism enabling *C. jeikeium* cells to speed up response time and react rapidly to changes in the environment.

#### Transcriptional control of additional lipid metabolism genes by GlxR and Jk0257

Recently, the involvement of the global regulator GlxR in the expression of further *fad* genes was proposed, due to the prediction of GlxR binding sites in their 5' regions (Barzantny *et al.*, 2012b). To demonstrate the interaction of GlxR and its predicted binding sites *in vitro*, the purified regulator was used for EMSA with fluorescently labelled PCR products comprising candidate regulatory regions of *fad* genes. These assays were separated by agarose gel electrophoresis and the retarded DNA-protein complexes were visualized by fluorescence imaging (Fig. 6). Eleven predicted binding sites were analysed, whereof nine showed a positive DNA band shift in the presence of cAMP, confirming the binding of GlxR to the upstream regions of *fadD3*, *fadD6*, *fadD7*, *fadD10*, *fadD11*, *fadE3*, *fadA1*, *fadA2*, and *fadH* (Fig. 6). In contrast, only two predicted target regions upstream of *fadD2* and *fadE7* did not interact with the purified GlxR protein *in vitro*, and can therefore be excluded from the GlxR regulon.



**Fig. 6.** DNA band shift assays with GlxR/Jk0257 and the upstream regions of selected *fad* genes. EMSA were conducted with the purified regulatory protein and fluorescently labelled PCR products carrying predicted GlxR binding sites. Assays were separated on 3% agarose gels and DNA-protein complexes were visualized by fluorescence imaging. Lanes 1, labelled PCR product; lanes 2, PCR product and regulatory protein; lanes 3, PCR product, GlxR and 0.6 mM cAMP.

To further investigate the co-regulatory role of Jk0257 in fatty acid metabolism, EMSA were conducted with the available fluorescently labelled PCR products and the purified Jk0257 protein (Fig. 6). In addition to the *fadE6* upstream region, Jk0257 is able to bind to the regulatory regions of the acyl-CoA synthetase genes *fadD2*, *fadD6*, *fadD10* and *fadD11*, the acyl-CoA dehydrogenase gene *fadE3*, the keto-acyl thiolase genes *fadA1* and *fadA2*, and the 2,4-dienoyl-CoA reductase gene *fadH*. In contrast, Jk0257 is unable to assemble with the upstream regions of *fadD3*, *fadD7* and *fadE7* *in vitro* (Fig. 6). The binding of Jk0257 without the addition of an effector molecule further supports the hypothesis that Jk0257 senses local signals and induces gene transcription by attenuated DNA binding, mediated by a specific ligand.

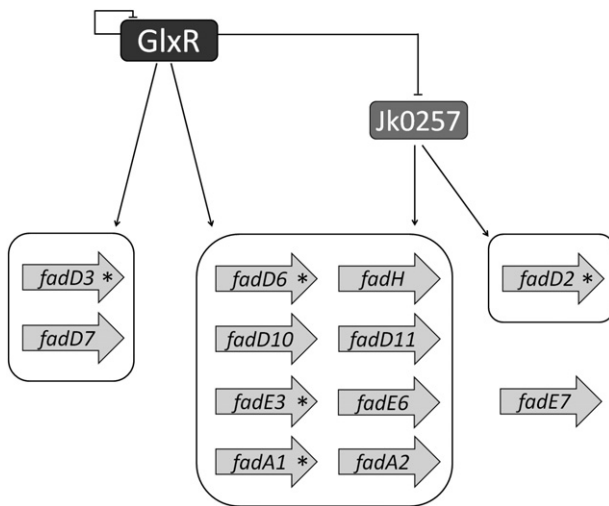
Taken together, DNA band shift assays demonstrate the co-regulation of eight *fad* genes by GlxR and Jk0257 (Fig. 7), affirming the involvement of GlxR and Jk0257 in

the coordinated regulation of fatty acid metabolism genes. Only a small proportion of analysed *fad* genes is regulated by GlxR (two genes) or Jk0257 (one gene) alone. Due to its apparent role in the regulation of fatty acid metabolism, we propose the name FamR for the MarR-like transcriptional regulator encoded by *jk0257* in *C. jeikeium*.

## Discussion

In this study, we demonstrated that the external lipid composition triggers the differential transcription of several lipid metabolism genes in the lipophilic skin resident *C. jeikeium*. A two- to ninefold induction of transcription was observed for *fad* genes during growth on Tween 60, clearly demonstrating the influence of external lipid sources on the control of *fad* gene expression. The degradation of skin lipids by lipophilic corynebacteria is one of four routes believed to contribute to the generation of





**Fig. 7.** Transcriptional control of *fad* genes by GlxR and Jk0257. The regulatory proteins GlxR and Jk0257 co-regulate the transcription of eight *fad* genes, including *fadE6*. Only three genes are not under dual control of GlxR and Jk0257. The *fadE7* gene was not assigned to either regulon. Arrows indicate a regulatory interaction; –| indicates repressive control mechanisms. Genes carrying a RamA binding site in their 5' region are marked with an asterisk.

human body odour (Taylor *et al.*, 2003; James *et al.*, 2004). Our data provide first evidence that the transcription of genes assigned to this route is susceptible to external lipid compounds, even though the essential enzymes of fatty acid degradation in *C. jeikeium* could hitherto not be identified in the annotated repertoire. However, the functional dissection of the multitude of paralogous Fad enzymes and their catalytic properties are currently under investigation.

DNA affinity chromatography and subsequent DNA band shift assays identified the global regulator GlxR and the MarR-like regulator Jk0257, now named FamR, as key regulators of *fad* gene transcription. GlxR controls the transcription of *fad* genes directly by binding the corresponding upstream regions and indirectly by regulating the transcription of *famR*. Consequently, our results show that the regulation of *fad* gene transcription is achieved through a hierarchical control mechanism in *C. jeikeium*, which is established by the global regulator GlxR in conjunction with FamR. A negative autoregulation of the *glxR* gene, previously also detected in the amino acid producer *C. glutamicum* (Jungwirth *et al.*, 2008), was demonstrated in *C. jeikeium* by DNA affinity chromatography and EMSA. GlxR is associated with the transcriptional control of diverse cellular functions and represents a global hub in the TRN of *C. jeikeium* (Barzantny *et al.*, 2012b). Hence, the TRN allows the synchronization of different metabolic pathways and the integration of global and local signals for the transcriptional control of lipid metabolism genes.

Additionally, RamA binding sites were predicted for several *fad* genes (Barzantny *et al.*, 2012b), possibly con-

necting the control of central carbon and lipid metabolism. RamA is an extensively characterized transcriptional regulator involved in the control of central carbohydrate metabolism in *C. glutamicum* (Auchter *et al.*, 2011). The presence of RamA binding sites implies that fatty acid metabolism is regulated on several levels by global, master and local regulators and points out the essential role of this metabolic pathway in the lipophilic species *C. jeikeium*. The involvement of three regulators demonstrates that tight regulatory mechanisms are necessary in this fatty acid auxotrophic organism for the adaptation of central metabolism to lipid-rich habitats. GlxR and RamA belong to the corynebacterial core set of transcriptional regulators (Barzantny *et al.*, 2012b) and probably play key regulatory roles in corynebacteria, characterized by diverse metabolic abilities (Barzantny *et al.*, 2012b). The assignment of *fad* genes to the GlxR regulon of *C. jeikeium* provides a new physiological function for this global regulator and supports the assumption that corynebacterial regulators show a higher level of conservation than the gene composition of their regulons (Barzantny *et al.*, 2012b). The evolution and adaptation processes of distinct species in the genus *Corynebacterium* are apparently facilitated by the variable genetic composition of their regulons.

The transcriptional regulator FamR was detected by DNA affinity chromatography with the upstream region of *fadE6* and shown to bind to the promoter region by EMSA. This regulator belongs to the MarR protein family, whose members are widely distributed among bacteria and archaea (Perera and Grove, 2010). Currently, about 12 000 MarR-like proteins have been reported, whereof only a hundred have been studied physiologically (Perera and Grove, 2010). Members seem to be involved in the control of diverse cellular processes, like the regulation of oxidative stress, the degradation of phenolic compounds, and virulence (Ellison and Miller, 2006; Lan *et al.*, 2010). Until now, no MarR homologue has been associated with the transcriptional control of lipid metabolism. We present the first data showing that the MarR-like regulator FamR can contribute to the adaptation of *C. jeikeium* to external lipids by regulating lipid metabolism genes. The topology of MarR-like proteins is highly conserved among different species including a winged helix–turn–helix motif and a specific ligand-binding pocket (Perera and Grove, 2010). The attenuated binding of MarR homologues by interaction with a specific ligand is characteristic of this group of regulators. MarR in *E. coli* responds to a range of anionic lipophilic compounds such as 2,4-dinitrophenol, menadione and salicylate (Aleksun and Levy, 1999; Perera and Grove, 2010), whereas the function of several MarR-like proteins is modulated by the ligand ureate (Perera and Grove, 2011). However, ligands for only a fraction of MarR-like regulators have been iden-

tified so far (Wilkinson and Grove, 2006). It is conceivable that FamR interacts with lipid substrates or intermediates from lipid metabolism and thereby responds to nutritional changes in the habitat of *C. jeikeium*. In this way, external signals, like changes of the lipid composition or the limitation of lipid substrates, can be detected and the *C. jeikeium* cell is able to react upon these signals with altered gene expression levels.

For several MarR-like regulators specific DNA-binding motifs have been identified, usually with palindromic character (Perera and Grove, 2010). However, the binding of highly degenerated palindromic sequences has been demonstrated for the MarR-like regulator PecS of *Erwinia chrysanthemi* (Rouanet *et al.*, 2004). DNA band shift assays narrowed a potential FamR binding site down to a 63 nt stretch downstream of the *fadE6* transcription start site. This position of a potential FamR binding sequence suggests a mechanism of road blocking for the transcriptional control of *fadE6*. However, a second possible FamR binding site was localized in a 251 bp fragment upstream of the *fadE6* promoter region. Therefore, DNA looping is probably also involved in the control of *fadE6* transcription, thereby enabling distant DNA regions and FamR proteins to interact with each other to coordinate gene activity. A candidate FamR binding motif (GATGTgA-CATC) was found in the *fadE6* upstream region at position -139 to -129 relative to the *fadE6* start codon. Interestingly, similar DNA motifs with the consensus sequence GRYSTGMAWYA were detected in the upstream regions of all genes positively tested for FamR binding, including DNA fragment 2 of the *fadE6* region (data not shown). In principle, MarR-like regulators are sequence-specific in their binding, although they are also known to associate with highly degenerated DNA motifs (Perera and Grove, 2011). Further research is necessary to investigate whether the detected DNA motifs are relevant for the transcriptional control of fatty acid metabolism genes in *C. jeikeium*.

The additional DNA band shift assays demonstrated the regulation of nine *fad* genes by FamR. This observation extended the physiological function of FamR in the regulation of fatty acid metabolism, as its controls not only the expression of the acyl-CoA dehydrogenase FadE6, but also the expression of FadE3, four acyl-CoA synthetases, two ketoacyl-CoA thiolases, and the 2,4-dienoyl-CoA reductase FadH. Consequently, FamR could be involved in the regulation of further genes associated with lipid metabolism in *C. jeikeium*. Interestingly, the degradation of lipid compounds is also associated with virulence of *C. jeikeium* (Tauch *et al.*, 2005), which prompts the question, whether FamR is also involved in the transcriptional control of virulence genes. Due to the restricted genetic accessibility of *C. jeikeium* and its multi-resistance profile, the analysis of a FamR knockout mutant is technically not

feasible. However, the application of global *in vivo* techniques like ChIP-Seq (Park, 2009) could facilitate the identification of additional members of the FamR regulon, thereby defining more precisely its physiological role in *C. jeikeium* and its relevance in the hierarchy and architecture of the TRN.

The metabolism of skin lipids by bacterial enzymes is a proposed route leading to the development of human axillary odour (Taylor *et al.*, 2003; James *et al.*, 2004). The elucidation of lipid metabolism in the model organism *C. jeikeium* can support the cosmetic industry in the process of developing new deodorant additives specifically targeting keyplayer enzymes. Efficient targeting of lipid metabolism in odour-causing bacteria could be investigated as soon as the pathway and the associated regulatory control mechanisms in lipophilic corynebacteria are fully understood. Our findings provide first evidence that the variation of external lipid sources induces an altered gene expression in lipophilic corynebacteria residing in the human axilla, possibly influencing bacterial lipid degradation, a process potentially connected to the generation of human body odour.

## Experimental procedures

### Bacterial strains and growth conditions

*Escherichia coli* DH5 $\alpha$ MCR (Grant *et al.*, 1990) and *E. coli* JM109 (Promega) as well as T7 Express Competent *E. coli* ER2566 (New England Biolabs) were routinely grown at 37°C in Luria–Bertani (LB) medium (Sambrook and Russel, 2001). The strains were used for standard cloning procedures and heterologous protein expression respectively. Selection for the presence of plasmids in *E. coli* was performed with ampicillin (100  $\mu$ g ml<sup>-1</sup>) and kanamycin (30  $\mu$ g ml<sup>-1</sup>). *Corynebacterium glutamicum* ATCC 13032 was routinely grown at 30°C in CASO bouillon (Roth). Plasmid selection was carried out with kanamycin (30  $\mu$ g ml<sup>-1</sup>). Cultures of *C. jeikeium* K411 (NCTC 11915) were grown at 37°C in BYT medium (Tauch *et al.*, 2004) or CJK minimal medium (Brune *et al.*, 2011) supplemented with 1% (v/v) Tween 80, Tween 60 or Tween 40. The growth of bacterial cultures was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>) with a BioPhotometer (Eppendorf).

### Standard DNA techniques

Plasmid DNA was prepared from *E. coli* cells by an alkaline lysis technique using the GeneJET Plasmid Mini Kit (Thermo Scientific). The restriction of DNA, analysis by agarose gel electrophoresis and DNA ligation were performed according to standard protocols (Sambrook and Russel, 2001). The transformation of *C. glutamicum* and *E. coli* cells was carried out by electroporation (Tauch *et al.*, 1994; 2002) and for the latter to some extent with chemocompetent cells. PCR assays were performed with chromosomal template DNA, a Tprofessional thermocycler (Whatman Biometra) and either Phusion DNA polymerase (Finnzymes) or BIOTAQ DNA

polymerase (Bioline). Chromosomal DNA was prepared from *C. jeikeium* cells with the Genomic-tip G/500 (Qiagen). PCR products were purified using the High Pure PCR purification kit (Roche Diagnostics) or the NucleoSpin PCR purification kit (Macherey-Nagel). All oligonucleotides used in this study were purchased from Metabion or Invitrogen and are listed in Table S2.

#### Detection of transcriptional start sites by 5' RACE-PCR

The preparation of total RNA from *C. jeikeium* cultures was performed as described previously (Brune *et al.*, 2006). RACE-PCR primers binding 20–200 bases downstream of the annotated start codon of *fadE6*, *jk0257* or *glxR* and 2 µg of total RNA were used for cDNA synthesis (Table S2). The resulting cDNA was modified and subsequently amplified by two additional gene-specific PCRs using the 5'/3' RACE Kit second generation (Roche Diagnostics). The PCR products were cloned into the pJET1.2/blunt vector (Thermo Scientific) and transferred into electrocompetent *E. coli* DH5αMCR or chemocompetent *E. coli* JM109. The sequencing of cloned RACE-PCR products was performed by IIT Biotech.

#### Detection of DNA-binding proteins by DNA affinity chromatography

To detect candidate regulatory proteins binding to the promoter regions of *fadE6*, *jk0257* and *glxR* from *C. jeikeium*, an experimental approach based on DNA affinity chromatography was performed. For the approach with *fadE6*, a protein raw extract was obtained from 50 ml cultures of *C. jeikeium* K411 grown in CJK minimal medium complemented with 1% (v/v) Tween 80, 60 or 40, whereas for the genes *jk0257* and *glxR*, an extract from a 50 ml BYT culture of *C. jeikeium* K411 was used. Cells were harvested at an OD<sub>600</sub> of 3.5 and 15 respectively. DNA fragments covering the 5' region of the three genes were generated by PCR using a biotin-modified primer pair. About 300 pmol of each amplified DNA fragment were immobilized at streptavidin-coated magnetic beads as recommended by the manufacturer (Chemagen). After three washing steps using DNA-binding buffer (50 mM Tris, 0.5 mM EDTA, 1 M NaCl; pH 7.5), the magnetic beads were resuspended in protein binding buffer [20 mM Tris, 1 mM EDTA, 10% (v/v) glycerol, 1 mM DTT, 100 mM NaCl, 0.05% (v/v) Triton X-100; pH 8.0] and incubated with the *C. jeikeium* protein extract in a rotator at room temperature for 1 h. The magnetic beads were subsequently washed three times with protein-binding buffer. Proteins bound to the immobilized DNA fragment were eluted with elution buffer (protein-binding buffer with 1 M NaCl). The eluted proteins were separated by SDS-PAGE and visualized by a Coomassie brilliant blue staining method. An in-gel digestion with modified trypsin (Promega) was carried out to identify single proteins bands. All mass spectrometric data were acquired using an ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). MS spectra (500–4000 m/z) were recorded in positive reflector mode with 3000 shots per MS spectrum. Precursor ions for MS/MS fragmentation were selected according to the results of the peptide mass fingerprint search of the MS spectrum. MS/MS spectra were processed using flexAnalysis (Bruker

Daltonics). The resulting peaklists were sent to an in-house Mascot server and searched against a database containing the predicted protein sequences of *C. jeikeium* K411. The search was performed choosing trypsin as enzyme with one missed cleavage allowed. As variable modifications carbamidomethyl (C) and oxidation (Met) were used. The peptide mass tolerance was set to ± 100 ppm and the fragment mass tolerance to ± 0.7 Da.

#### Purification of the GlxR and the Jk0257 protein

The Intein Mediated Purification with an Affinity Chitin-binding Tag (IMPACT) system (New England Biolabs) was applied to purify the GlxR and the Jk0257 proteins of *C. jeikeium* K411. The *glxR* and *jk0257* coding regions, with the exception of the stop codon, were amplified by PCR using the primer pairs *jk1972\_NdeI/jk1972\_SapI* and *jk0257\_NdeI/jk0257\_SapI* (Table S2) respectively. For Jk0257 protein expression, an alanine codon was added at the 3' end of the coding region in order to minimize *in vivo* splicing of the intein-tagged protein due to the native C-terminal asparagine residue. Additionally, an NdeI site and a SapI site were added to the 3' and 5' ends of the resulting DNA fragment respectively. The amplified DNA fragments were digested with NdeI and SapI and cloned in *E. coli* DH5MCR into the vector pTXB1 (New England Biolabs). The resulting plasmids pTXB1-*jk0257* and pTXB1-*jk1972* were transferred into the expression strain *E. coli* ER2566 (New England Biolabs) by electroporation. *Escherichia coli* ER2566 [pTXB1-*jk0257*] and [pTXB1-*jk1972*] were grown under selective conditions in LB medium. At an OD<sub>600</sub> between 0.5 and 0.8, a final concentration of 0.4 mM IPTG was added to the culture and the cultivation temperature was reduced to 30°C. After 4 h of growth at 30°C, the *E. coli* cells were harvested by centrifugation at 2790 g and 4°C for 15 min. Cell pellets were resuspended in pre-chilled lysis buffer (20 mM Tris, 500 mM NaCl; pH 8.5) complemented with the protease inhibitor cocktail cComplete Mini (Roche Diagnostics). Cells were disrupted twice at a speed of 6500 r.p.m. for 30 s using a Precellys 24 Homogenizer (Peqlab) and ribotubes, containing glass beads with a diameter of 150 to 212 µm (Sigma-Aldrich). After cell disruption the samples were centrifuged (17 949 g, 4°C, 30 min) to remove all cell debris and the supernatant was treated with Benzonase Nuclease (Sigma-Aldrich) at 4°C for 30 min. The assay was cleared by centrifugation at 2790 g and 4°C for 15 min. Further experimental steps were carried out according to the IMPACT protocol (New England Biolabs) using a modified washing buffer (20 mM Tris, 1 M NaCl). The cleavage reaction was performed at 4°C for 48 h. The resulting protein solution was concentrated by applying Amicon ultra-4 10 000 MWCO centrifugal filter units (Millipore). To optimize the protein stability of Jk0257 and GlxR, a washing step with phosphate buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM NaCl, 1 mM EDTA) and GlxR-binding buffer (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl, 2.7 mM KCl; pH 8.5) was appended respectively. The concentrated and washed GlxR and Jk0257 proteins were visualized by SDS-PAGE on a 12.5% gel and finally stored at -20°C and +4°C respectively. The protein concentration was determined with the Bio-Rad protein assay kit. The purification of the regulatory proteins was verified by peptide mass fingerprinting.

*EMSA with the purified GlxR and Jk0257 protein*

The EMSA were performed with Cy3- or fluoresceine-labelled PCR products. During DNA band shift assays, 60–80 pmol of the purified GlxR or 10–30 pmol Jk0257 protein were mixed with 0.1 pmol labelled PCR product, 1–2  $\mu$ l herring sperm DNA (0.1  $\mu$ g ml<sup>-1</sup>), 15% (v/v) glycerol and GlxR-binding buffer or Jk0257-binding buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 75 mM KCl, 0.1 mM EDTA) to get a total volume of 20  $\mu$ l. The GlxR assays were incubated at 37°C for 30 min and then separated in 3% agarose gels prepared in gel buffer (40 mM Tris, 1 mM sodium acetate, 1 mM EDTA). The Jk0257 assays were incubated at room temperature for 20 min and then separated in 3% agarose gels prepared in gel buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>). A voltage of 70 V was supplied for 90 min. The agarose gels were scanned with a Typhoon 8600 Variable Mode Imager (GE Healthcare).

*Testing the regulator binding activity of Jk0257 in vivo*

The upstream region of *fadE6* carrying the mapped promoter region was amplified by PCR with the primer pair *fadE6\_Prom\_fwd/rev* (Table S2). The product was digested with the restriction enzymes NsiI/BamHI and ligated into the vector pEPR1 (Knoppová *et al.*, 2007) upstream of a promoterless gene encoding the green fluorescent protein (Gfp), resulting in the vector pEPR1-*fadE6*. The gene *jk0257* was amplified by PCR with the primer pair *jk0257\_Gfp\_a/b* (Table S2), resulting in a 699 bp fragment carrying also the native *jk0257* promoter region. To analyse the *in vivo* binding activity of Jk0257 to the *fadE6* promoter region, the 699 bp fragment was cloned into the vector pEPR1-*fadE6* (digested with SmaI), resulting in the vector pEPR1-*fadE6-jk0257*. A clone was selected carrying the *jk0257* gene and its promoter divergently oriented to the *fadE6* promoter, ensuring a distance of 85 bp between the -35 regions of both promoters for sufficient RNA polymerase binding. For the analysis of *gfp* expression the vectors pEPR1, pEPR1-*fadE6* and pEPR1-*fadE6-jk0257* were transformed separately into *C. glutamicum* ATCC 13032 cells by electroporation. All strains were cultivated as described, harvested in the exponential growth phase at an OD<sub>600</sub> of 4.0, washed twice with PBS buffer (20 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4) and diluted to an OD<sub>600</sub> of 0.3. Fluorescence measurements were carried out at 385/509 nm with the Tecan Infinite 200 PRO in black 96-well polystyrene microplates (Greiner bio-one).

**Conflict of interest**

None declared.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Plasmids used in this study.

**Table S2.** Primer sequences used in this study.