On the enigma of glutathione dependent styrene degradation in *Gordonia rubripertincta* CWB2

Thomas Heine[1,#], Juliane Zimmerling[1], Anne Ballmann[1], Sebastian Bruno Kleeberg[1], Christian Rückert[2], Tobias Busche[2], Anika Winkler[2], Jörn Kalinowski[2], Ansgar Poetsch[3,4], Anika Scholtissek[1], Michel Oelschlägel[1], Gert Schmidt[5] and Dirk Tischler[1,6,#]

[1]Institute of Biosciences, TU Bergakademie Freiberg, Leipziger Str. 29, Freiberg, Germany.

[2]Technologieplattform Genomik, Centrum für Biotechnologie (CeBiTec), Universität Bielefeld, Universitätsstr. 27, Bielefeld, Germany.

[3]Plant Biochemistry, Ruhr University Bochum, 44801 Bochum, Germany

[4]School of Biomedical and Healthcare Sciences, Plymouth University, Plymouth PL4 8AA, UK

[5]Institut für Keramik, Glas- und Baustofftechnik, TU Bergakademie Freiberg, Agricolastr. 17, Freiberg, Germany.

[6]Microbial Biotechnology, Ruhr University Bochum, 44801 Bochum, Germany

[#]For correspondence. E-mail heinet@tu-freiberg.de, dirk-tischler@email.de; Tel. +49 3731 392103.

Running title: Styrene degradation via glutathione-*S*-transferase

SUPPLEMENTAL MATERIAL

Description and Growth of *Gordonia rubripertincta* CWB2

The strain CWB2 belongs to the genus *Gordonia* which exists in various native biotopes but also in artificial habitats as well as the human body (1). These actinomycetes are of interest for biotechnological applications and organic synthesis as they are able to degrade various pollutants and xenobiotic compounds (2). 16S rRNA phylogenetic analysis (Fig. S1) and *in silico* DNA-DNA-hybridization (> 80% similarity with *G. rubripertincta* NBRC 101908; Fig. S7) classifies strain CWB2 within the species *Gordonia rubripertincta*. The complete closed genome of strain CWB2 consists of a chromosome with a size of 5.23 Mbp and a GC content of 67.2%, which is in accordance with the species strain. Further, strain CWB2 harbours one plasmid (pGCWB2) with a size of about 100 kbp and a GC content of 64.2% (Table S3).

G. rubripertincta CWB2 is Gram-positive, catalase positive and able to produce siderophores under iron-limitation but does not synthesize surfactants. The mycolic acid composition is: C_{53}: 6%, C_{54}: 7%, C_{55}: 18%, C_{56}: 17%, C_{57}: 25%, C_{58}: 12%, C_{59}: 10%, C_{60}: 4%, C_{61}: 1%. The fatty acid profile is shown in Table S2. Most abundant fatty acids are palmitic acid, oleic acid, palmitoleic acid/2-hydroxy-14-methylpentadecanoic acid and tuberculostearic acid. Strain CWB2 is resistant or not affected by nalidixic acid, slightly sensitive to ampicillin, gentamycin and tetracycline and highly sensitive to chloramphenicol and kanamycin (no growth). Further, strain CWB2 seems to be able to develop a resistance mechanism against streptomycin. It produces a rose pigment and forms aggregates during growth. However, the colour intensity and size of the agglomerates can differ depending on the substrate.

G. rubripertincta CWB2 is able utilize a variety of carbon and energy sources like sugars, amino acids, organic acids, aromatic acids and other xenobiotics for growth

(Table S1). Thus, strain CWB2 has a broad substrate spectrum even if compared to other *Gordonia* species (3, 4). However, *G. rubripertincta* CWB2 is the second reported representative of this genus that is able to degrade and withstand high amounts of the hazardous chemical styrene (5–7). Initial studies showed that it is able to metabolize about 0.2 $mg_{styrene}\ h^{-1}mg_{cdw}^{-1}$. Under these conditions strain CWB2 forms rod shaped cells with a length between 817 – 1940 nm and a diameter of 218 – 520 nm. When grown on fructose, its cells form cocci with an average length between 946 – 1290 nm and a diameter of 620 – 671 nm (Fig. S3). It inclines to form aggregates on both substrates that can reach up to 65 µm. In particular, strain CWB2 was cultivated on different substrates that are in relation with the styrene degradation pathways, isoprene degradation, metabolites that are part of the TCA cycle and fructose as reference (Table 1). Strain CWB2 shows good growth on all of the metabolites from the upper styrene degradation pathway. However, a comparable growth to fructose is only seen with phenylacetic acid. No growth was observed when strain CWB2 is exposed to isoprene or mandelic acid as sole carbon source.

Microbial strategies for degradation of styrene.

Natural sources of styrene as well as anthropogenic emission into the environment make it available for microorganisms as source for carbon and energy. The capability to degrade styrene has been described for many microorganisms. Therefore, they have evolved different strategies to attack the molecule. Direct ring cleavage is proposed to occur unspecifically via enzymes that are relevant for degradation of analogous aromatic substrates like benzene, toluene and catechol (8–10).

Attack at the vinyl side chain is the only styrene specific degradation pathway and seems to be favoured by microorganisms (11). Styrene is initially converted to

styrene oxide. Aside from the SMO, other monooxygenases are known to be able to epoxidize styrene (e.g. binuclear iron monooxygenases (BIMO) and CYP450MOs) (12–17). Thereby, especially the binuclear iron monooxygenase might play a role. These enzymes are known to be able to oxidize a variety of organic compounds and in particular to epoxidize alkenes (18). The respective cluster contains a chaperone, which is necessary for successful synthesis of the monooxygenase complex (19). It was recently shown that expression of a homologous BIMO in *Gordonia* sp. i37 is (co-)induced by isoprene (20). Although no evidence was found there, that the BIMO is involved in isoprene epoxidation, several studies support that styrene can serve as a substrate for these monooxygenases (12, 15, 21).

Interestingly, SOIs which catalyze the next step of styrene degradation, the isomerization of styrene oxide to phenylacetaldehyde, seem to be less abundant in nature. This phenomenon was observed in a screening of the non-redundant protein database as well as of the strain collection of the Institute of Biosciences at the TU Bergakademie Freiberg (6). Herein, only 14 out of 87 selected styrene degraders showed SOI activity. As a consequence, several other metabolites than the SOI product phenylacetaldehyde were consistently detected during growth on styrene (e.g. 2-phenylethanol). This implies that other enzymes are involved in styrene degradation (22–25) and it is likely that side-chain attack without SOI is more common as assumed.

It was shown for *Pseudomonas* sp. Y2 that the upper and lower pathway can be connected via regulatory elements (26). The complete regulatory, uptake and metabolization machinery has only been described in detail for pseudomonads so far (reviewed by (11) but cluster analysis indicate deviating regulation in other phyla. Besides the key elements for styrene degradation (StyABCD), regulators and

transporters are not conserved and the genetic organization differs among organisms (6, 27–31).

Remarks on Unspecific Styrene Degradation Pathways

In humans and mammals, styrene is epoxidized to styrene oxide by cytochrome P450 monooxygenases (CYP450MO) (32). This reactive intermediate forms glutathione conjugates by the activity of a glutathione *S*-transferase (GST) or is converted to styrene glycol by an epoxide hydrolase (EH) (33, 34). The glycol can be further metabolized to mandelic acid and phenylglyoxylic acid. The metabolites are subsequently excreted (32, 35). Similar to this route, some white-rot fungi are able to further catabolize glycol to benzoic acid and minor amounts of 2-phenylethanol (36).

2-Phenylethanol can emerge as a side product of styrene degradation routes (25, 36) but is also proposed to be a central metabolite in several cases (22–24, 37, 38).

Several unspecific routes for styrene degradation are employed by microorganisms. These routes are supposed to reflect altered utilization of benzene-, toluene- and ethylbenzene degradation pathways (8, 39–41). Herein the aromatic nucleus is attacked by a styrene 2,3-dioxygenase (SDO) and a styrene 2,3-dihydrodiol dehydrogenase (SDD) to yield 3-vinylcatechol. The 3-vinylcatechol can undergo *ortho*- or *meta*-cleavage as found in *Rhodococcus rhodochrous* NCIMB 13259 (8, 42). Though, *ortho*-cleavage by the activity of a vinylcatechol-1,2-dioxygenase (VC12DO) leads to a dead-end product. On the other hand, complete metabolization of styrene by *meta*-cleavage is reported. A vinylcatechol-2,3-dioxygenase produces 2-hydroxy-6-oxo-octa-2,4,7-trienoic acid which is further converted into acrylic acid, acetaldehyde and pyruvate (8, 10, 40). A variation of this pathway was found in *Pseudomonas* sp. Y2 (24).

Additional information to the situation in strain CWB2.

Degradation pathways via direct ring cleavage might also be possible in strain CWB2 as related genes were also found within the genome. A vinylcatechol 1,2-dioxygenase can be found twice on the genome (GCWB2_07645; GCWB2_20650). The latter one is part of a cluster including enzymes for benzoate and catechol degradation at the *ortho* position. However, the activity of the dioxygenase leads to a dead-end product and no further metabolization via the *ortho*-cleavage pathway is known for styrene so far. Further, a styrene dioxygenase (SDO) or biphenyl 2,3-dioxygenase is not encoded on the genome and thus, the initial step for the ring attack is lacking (10). Absence of a vinylcatechol 2,3-dioxygenase prevents degradation via the *meta*-cleavage pathway.

It is known for some organisms that styrene oxide can be further metabolized by an epoxide hydrolase (EH) to styrene glycol, which is further degraded by other enzymes to mandelic acid and benzoic acid. An EH, which is similar to that of *Sphingomonas* sp. HXN-200 (ANJ44372), can be found in strain CWB2 (GCWB2_06100 – 48%) (43). Four other putative EHs are encoded and one of them lies close to the styrene degradation cluster on the plasmid (GCWB2_24135).

However, none of the above mentioned genes are upregulated on transcriptome level and none of the respective proteins were detected under styrene exposure. Therefore, it is unlikely that these are involved in styrene degradation.

Proteome data of the upregulated clusters show that most of the proteins were detected in the cytosolic and the membrane fraction. However, the difference between both fractions can be an indication for the subcellular localization. Especially proteins that are supposed to be membrane bound are more abundant in the membrane fraction. Further, it might be reasonable that some of the cytosolic

proteins are also situated at the membrane boundary as styrene might also accumulate at there. Some of the proteins (e.g. PaaD and PaaI) were not detected in the fructose reference and therefore no ratio can be calculated (Table 2 and Dataset S1).

Interestingly, genes of cluster S3 and S4 are also expressed to some extend when strain CWB2 is cultivated under fructose as well as other conditions, what indicates for constitutive expression of these genes (unpublished data).

Beyond the styrene degradation cluster on pGCWB2, several other clusters are upregulated on RNA and/or protein level (Fig. S5 and S6, Dataset S1). This is true for the gene cluster containing the BIMO-like enzyme as well as the alkyl hydroperoxide reductase cluster. Two CYP450MO clusters are upregulated on protein level but not on RNA level. Therefore, strain CWB2 might employ a BIMO and two CYP450-like monooxygenases that are upregulated on protein level, to support the formation of styrene oxide (See SI for further information) (12, 13, 15, 16, 44–46). In contrast, the CYP450-like monooxygenases are downregulated on RNA and further investigations are needed to reveal if they play a functional role in styrene metabolization in strain CWB2.

Styrene is supposed to be a stressor for bacterial cells (47). Additionally, reactive oxygen species are continuously produced during aerobic metabolism. To prevent oxidative stress, strain CWB2 express and synthesises an alkyl hydroperoxide reductase (AHR, GCWB2_14195 - GCWB2_14185) to detoxify hydrogen peroxide, which is produced during aerobic metabolism of styrene (48–51). Furthermore, AHR might act as a glutathione peroxidase to reduce organic hydroperoxides to the corresponding alcohol and oxidized glutathione (52, 53).

A cluster which contains a coniferyl alcohol dehydrogenase and an aldehyde

dehydrogenase is somewhat downregulated in the transcriptome but clearly upregulated on protein level. Coniferyl alcohol can be metabolized via an aldehyde to cinnamic acid. As these have distinct structural resemblance with metabolites of the upper styrene degradation pathway, this cluster might be co-induced by these metabolites.

As already mentioned, pigment formation in strain CWB2 is substrate dependent. In accordance with that, the cluster for biosynthesis of ζ-carotene is downregulated under styrene exposure.

Remark on the isolation of *G. rubripertincta* CWB2 and other styrene degraders. As previously described, strain CWB2 was obtained from a soil sample (BioProject Accession: PRJNA394617; sampling location: 50°55'30.0"N 13°19'60.0"E) taken from potting soil of banana plant at the organic chemistry institute of the TU Bergakademie Freiberg (Saxony, Germany). It was designated according the Clemens-Winkler-Building, separated via styrene-enrichment culture and repeated platting on minimal medium with styrene as sole source of carbon and energy (6, 7, 54). The strain was deposited at DSMZ (Braunschweig, DSM 46758, (6, 7). All further strains were available at the Institute of Biosciences (TU Bergakademie Freiberg) or isolated and cultivated as described previously (6).

FIGURES

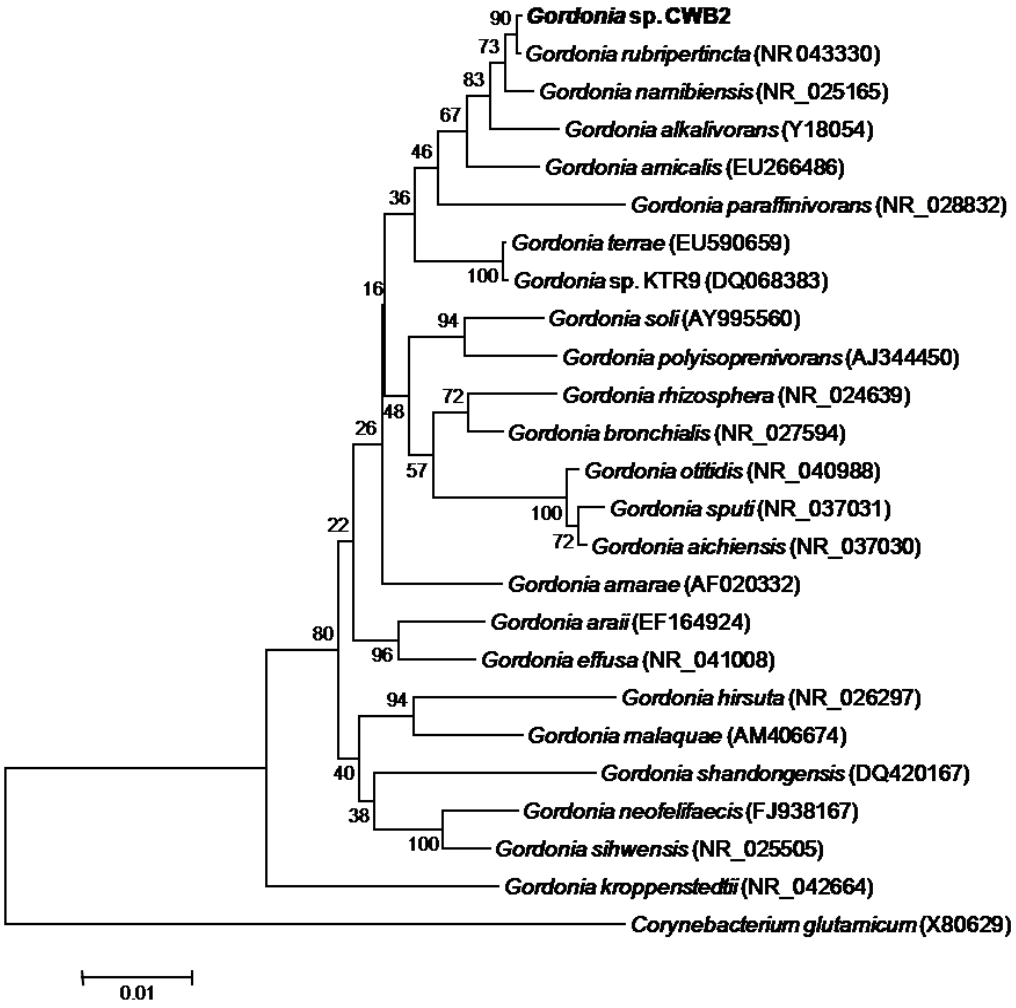

Fig. S1. Phylogenetic tree of selected strains of the genus *Gordonia*. 16S rRNA based neighbor-joining tree was calculated by using the MEGA7 software and a sequence alignment that was generated by ClustalW. *Corynebacterium glutamicum* served as outgroup. Bootstraps values were calculated by 1000 replicates and are given ad the nodes of the branches.

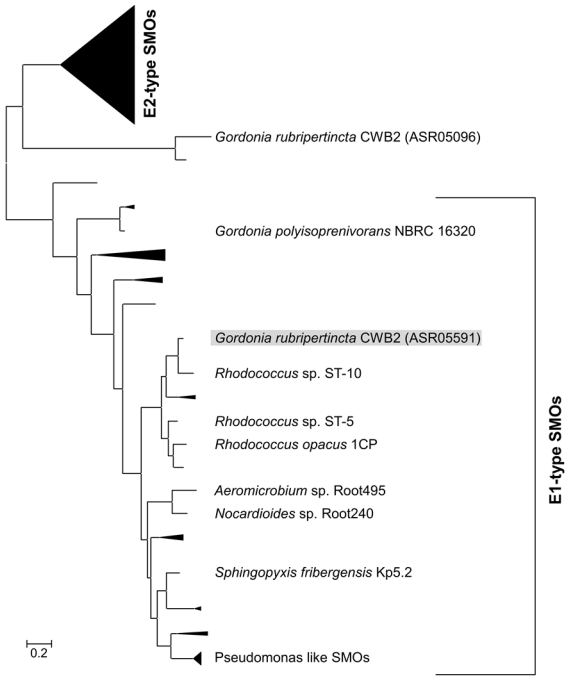

Fig. S2. Phylogenetic analysis of styrene monooxygenases (SMOs) with focus on ones used in this study. The active and upregulated SMO of *Gordonia rubripertincta* CWB2 is shaded grey. Amino acid sequences were aligned by using the ClustalW algorithm and the maximum likelihood tree was constructed by applying the MEGA7 software (55).

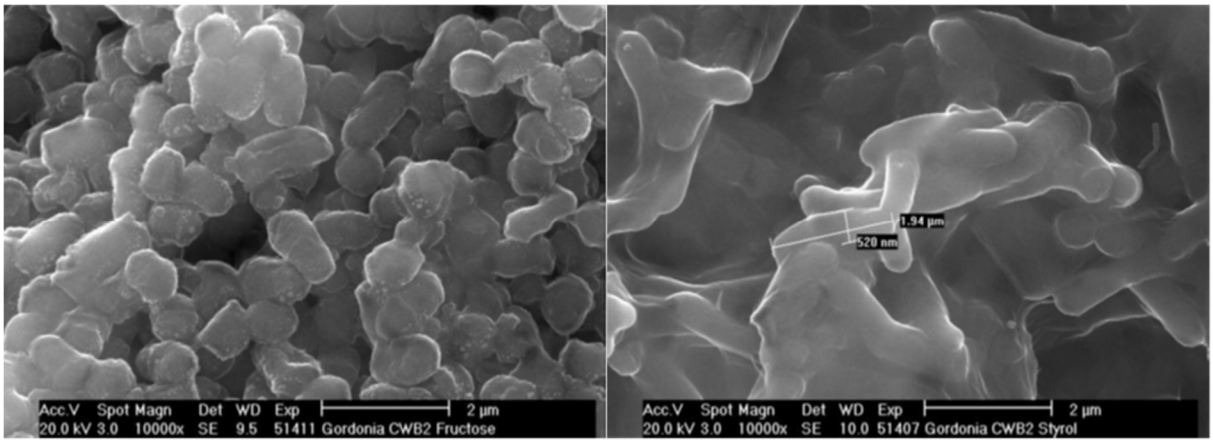

Fig. S3. Scanning electron microscopy (SEM) pictures of *G. rubripertincta* CWB2 cells after growth on fructose (left) or styrene (right).

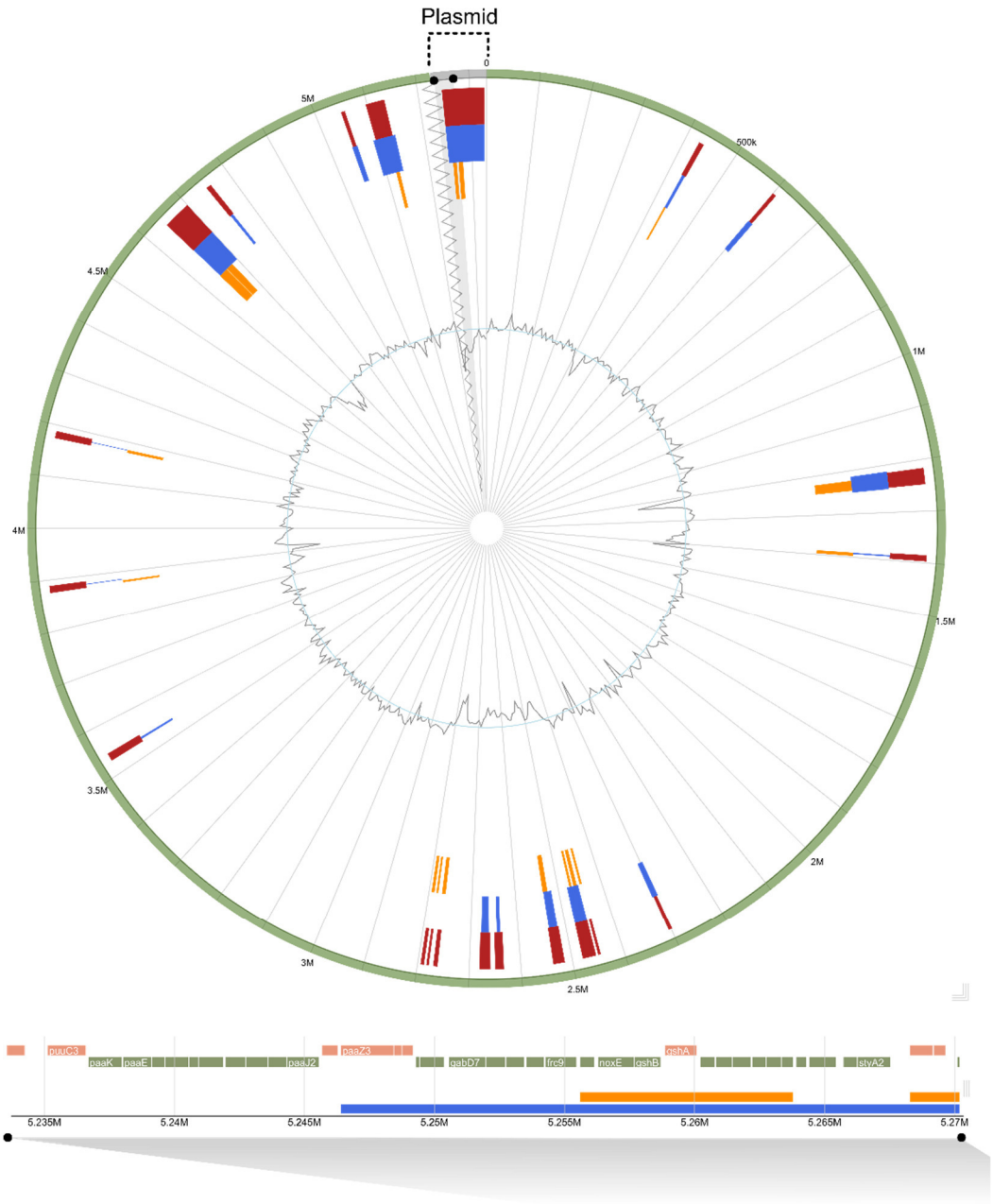

Fig. S4. Genomic island plot of the genome of *G. rubripertincta* CWB2. Analysis was done by IslandViewer 4 and genomic island predictions are represented as blocks (integrated results in red, SIGI-HMM in orange and IslandPath-DIMOB in blue). The genome of strain CWB2 was aligned against a reference *Gordonia* sp. KTR9. Aligned regions are indicated in green at the outer circle, while the unaligned region is shaded in grey and refers to the plasmid pGCWB2. The styrene degradation cluster is highlighted below. GC content is visualized within the genome plot.

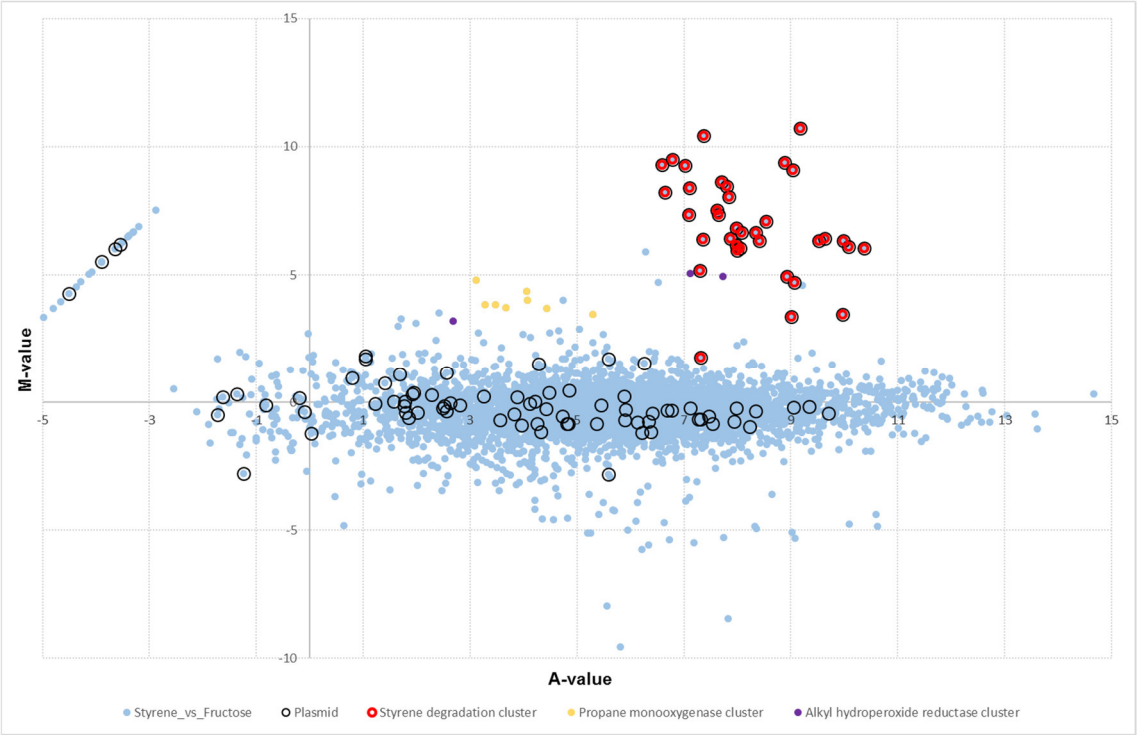

Fig. S5. Ratio/intensity (m/a) plot of the gene expression of styrene and fructose grown cultures of *Gordonia rubripertincta* CWB2. CDS with m-values of higher/equal than +1.5 or lower/equal than −1.5 were considered to be differentially transcribed.

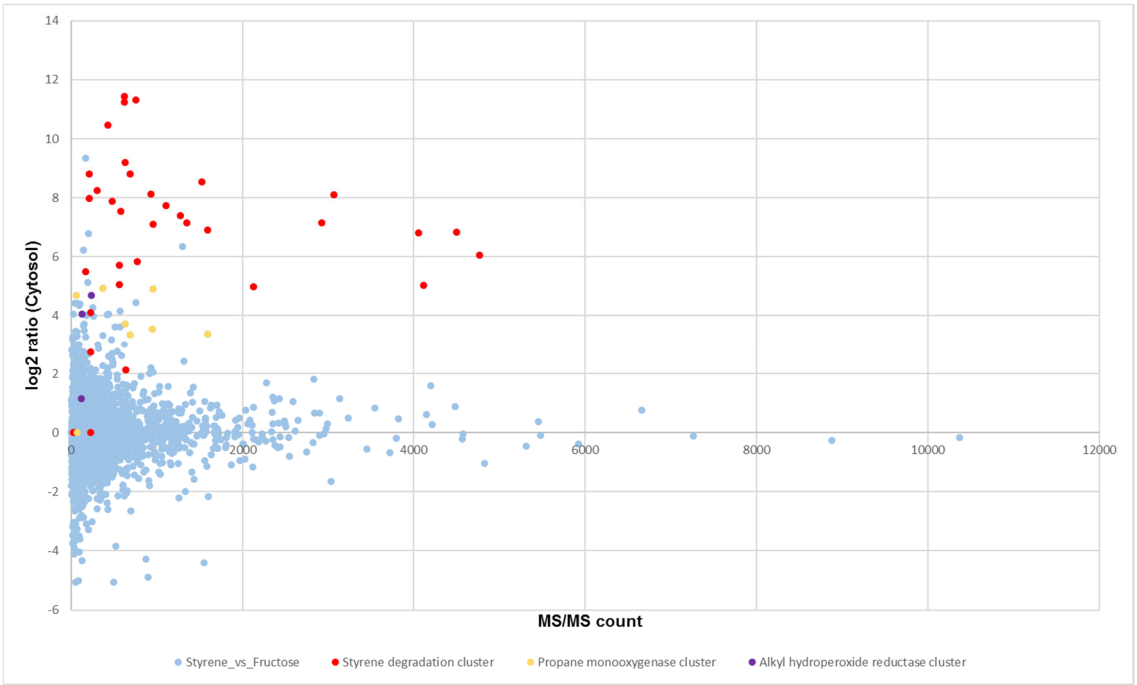

Fig. S6. Ratio/intensity (log2 ratio / MS/MS count) plot of the proteome (cytosolic fraction) of styrene and fructose grown cultures of *Gordonia rubripertincta* CWB2.

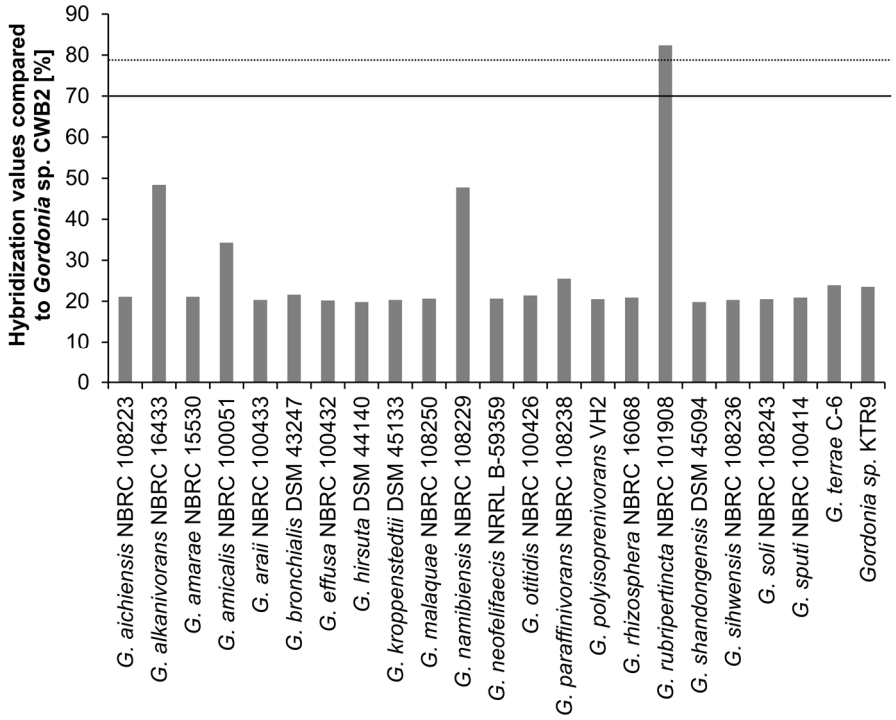

Fig. S7. *In silicio* genome-to-genome DNA-DNA hybridization of *Gordonia rubripertincta* CWB2 compared to representatives of *Gordonia* species. Thresholds of 70% (solid line; strains belong to the same species; 56) and 79% (dashed line; strains belong to the same subspecies; 57) are indicated.

TABLES

Table S1. Detailed substrate spectra of *G. rubripertincta* CWB2.

Substrate	Utilization	Substrate	Utilization	Substrate	Utilization
D-(+)-Glucose	++	L-Serine	+	Niacin	+
D-(-)-Fructose	+++	L-Alanine	++	Isoprene	-
D-(+)-Galactose	-	Aspartate	±	Styrene	++
D-(+)-Maltose	+	Succinate	++	Styrene oxide	++
L-(+)-Rhamnose	±	Citrate	++	Phenylacetaldehyde	++
D-Ribose	+	Sodium gluconate	+++	Phenylacetic acid	+++
D-(+)-Saccharose	+++	*alpha*-Ketoglutarate	++	2-Phenylethanol	++
D-Turanose	+++	Benzoate	++	*para*-Cresol	++
Trehalose	++	*ortho*-hydroxybenzoate	+	Biphenyl	+
Lactose	-	*meta*-hydroxybenzoate	+	Naphtalene	+
D-Arabitol	++	*para*-hydroxybenzoate	+	Phenanthrene	-
D-Mannitol	+++	*meta*-aminobenzoate	+	Hexadecane	++
myo-Inositol	+	*para*-aminobenzoate	+	Dodecane	+++
Glycerol	+++	Anthranilate	+	Tetradecane	++
N-acetylglucosamine	+	Mandelic acid	-	Decanoic acid	±
L-Leucine	+	*trans*-ferulate	+	*gamma*-Aminobutyrate	±
L-Proline	+	*trans*-cinnamate	+++	Pimelate	+++
L-Valine	+	Vanillate	+	Soluble starch	±

(+++) vigorous growth; (++) good growth; (+) growth; (±) marginal growth (-) no growth

Table S2. Fatty acid composition of *G. rubripertincta* CWB2.

Fatty acid	%	Fatty acid	%
Saturated		**Unsaturated**	
$C_{9:0}$	0.09	$C_{15:1}\omega 8c$	0.57
$C_{12:0}$	0.07	$C_{15:1}\omega 5c$	0.56
$C_{13:0}$	0.09	$C_{16:1}\omega 9c$	0.66
$C_{14:0}$	2.16	$C_{17:1}\omega 8c$	5.53
$C_{15:0}$	3.43	$C_{18:1}\omega 9c$	22.30
$C_{16:0}$	28.24	**Summed features**	
$C_{17:0}$	6.10	iso-$C_{15:0}$ 2-OH / $C_{16:1}\omega 7c$	12.55
10-Methyl $C_{17:0}$	1.19	$C_{19:1}\omega 9c$ / $C_{19:1}\omega 11c$	3.23
$C_{18:0}$	1.04	n.d.	0.23
10-Methyl $C_{18:0}$	11.82		
10-Methyl $C_{19:0}$	0.14		

n.d. – not determined

Table S3. Genome statistics of *Gordonia rubripertincta* CWB2

Attribute	Chromosome	Plasmid pGCWB2
Accession	CP022580	CP022581
Size (bp)	5,227,013	105,060
G + C content (%)	67.3	64.2
CDS	4,708	112
rRNAs (operons)	12 (4)	0
tRNAs	52	0
other RNAs	15	

Table S4. Summary of transcript reads of *Gordonia rubripertincta* CWB2

	Styrene	Fructose
Total reads	122 515 232	11 626 146
mapped reads	112 691 546	11 512 126
multiple mapped reads	3 504 830	90 879

Table S5. Activities of wild-type enzymes that are related to styrene degradation (given in U mg^{-1})

	SMO	SOR	PAR		PAD			VC12DO	GST
S	**ST***	**SO***	**2PE**	**SO***	**PA**	**SO***	**2PE***	**CA**	**SO***
CE	0.19×10^{-3}	6.21×10^{-5}	-	0.38	-	1.10×10^{-2}	2.18×10^{-2}	0.12	44.23
IE	0.42×10^{-3}	-	1.27×10^{-5}	-	3.72×10^{-5}	-	-	0.63	-
HI	6.82×10^{-3}	-	n.d.	-	n.d.	-	-	2.4	-
GF	-	-	-	-	-	-	-	3.82	-

CE - crude extract, IE - ion exchange, HI - hydrophobic interaction, GF - gel filtration, S - substrate, ST - styrene, SO - styrene oxide, PA - phenylacetaldehyde, 2PE - 2-phenylethanol, CA - catechol, n.d. - not detectable;
*determined by product quantification on RP-HPLC
Enzyme activities are given in 1 U mg^{-1} representing the conversion µmol substrate per min per mg protein.

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