Fluoride resistance in Streptococcus mutans

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Fluoride wordt al meer dan vijf decennia lang gebruikt als het meest effectieve anti-cariës agens. Langdurige blootstelling aan hoge concentraties fluoride kan leiden tot de ontwikkeling van fluorideresistentie van orale bacteriën. Dit proefschrift beschrijft een aantal experimenten die gericht zijn op fenotypische en genotypische kenmerken van fluorideresistente *Streptococcus mutans* stammen.

In hoofdstuk 2 werden deze kenmerken ondezocht voor de fluorideresistente stam *S. mutans* C180-2FR en zijn moederstam C180-2. *S. mutans* C180-2FR had een significant groter groeivermogen in aanwezigheid van fluoride dan C180-2. De kolonies van deze twee stammen vertoonden ook een duidelijk verschillende morfologie. Vervolgens hebben we "whole genome shotgun (WGS) sequencing" toegepast op deze twee stammen om genen te vinden die gerelateerd zijn aan fluorideresistentie. De hele genoomsequenties van deze twee stammen werden vervolgens met elkaar vergeleken. Op deze manier werden enkel-nucleotide polymorfismen (SNPs: single nucleotide polymorphisms) in het genoom van *S. mutans* C180-2FR geïdentificeerd, waarna deze bevestigd werden met traditionele Sangersequencing. De functies van deze genomische veranderingen werd verder onderzocht door de expressie te meten van de genen die één of meer SNPs bevatten. Een vergelijking tussen de genomen van beide stammen onthulde acht mutaties in C180-2FR, waarvan vijf SNPs gelegen waren in een gen en twee in intergene gebieden. Genexpressiegegevens toonden aan dat drie genen, stroomafwaarts van een promotor die een SNP bevatte, constitutief hoger tot expressie kwamen in C180-2FR in vergelijking met C180-2. Interessant genoeg zijn twee van de drie genen, *perA* en *perB*, homologen van *eriC*^{F}, een gen dat codeert voor een fluoridetransporteiwit. Deze studie toonde aan dat WGS sequencing een bruikbare methode is om veranderingen in het genoom van fluorideresistente *S. mutans* stammen te ontdekken.

Het verband tussen de mutatie in de promotor (*mutp*) van de genen coderend voor de fluoride-transport eiwitten en fluorideresistentie werd verder bestudeerd in **hoofdstuk 3**. Van de fluoridegevoelige stam *S. mutans* UA159 is een afgeleide stam geconstrueerd met de betreffende mutatie in *mutp*. Deze afgeleide stam, UF35 genaamd, werd gekenmerkt door zijn vermogen om in aanwezigheid van fluoride zowel te groeien als fermentatief melkzuur te produceren. De resultaten toonden aan dat deze afgeleide stam, UF35, kon groeien in aanwezigheid van hogere concentraties fluoride dan *S. mutans* UA159. In aanwezigheid van fluoride produceerde UF35 significant meer

melkzuur dan UA159. Echter, de zuurtolerantieresponstest toonde aan dat de mutant gevoeliger was voor zuurstress dan de wild-type stam. Deze verhoogde gevoeligheid van UF35 voor een lage pH kan het gevolg zijn van een energieverspillende "futiele protoncyclus" gemedieerd door de fluoridetransporteiwitten. Het effect van de mutatie op de activiteit van *mutp* werd bepaald door, zowel de expressie van stroomafwaarts gelegen genen, als de fluorescentie van reporterstammen te kwantificeren. Resultaten van beide bepalingen bevestigden dat de gemuteerde *mutp* constitutief meer actief was dan het wild-type *mutp*. Wij concludeerden dat de SNP in *mutp* de promotoractiviteit en de expressie van de fluoride-transport eiwitten verhoogt, resulterend in een verhoogde fluorideresistentie.

Gegevens uit hoofdstuk 3 bevestigden de rol van één van de acht mutaties die in hoofdstuk 2 waren gevonden. Hoewel de rol van de export van fluoride als een fluorideresistentie mechanisme voor de hand ligt, is het waarschijnlijk niet het enige mechanisme dat bacteriën gebruiken om fluorideresistent te worden. Eerdere studies hebben aangetoond dat dezelfde fluoridegevoelige stammen verschillende niveaus van fluorideresistentie kunnen ontwikkelen, wat kan wijzen op de betrokkenheid van meerdere genen. Om andere factoren die verband houden met fluorideresistentie te identificeren, werden in **hoofdstuk 4** de genoomsequenties van twee natuurlijk geselecteerde fluorideresistente stammen (UA159FR en C180-2FR) en die van hun moederstammen (UA159 en C180-2) geanalyseerd. Op deze manier werden in beide fluorideresistente stammen mutaties gevonden in dezelfde chromosomale gebieden. De genexpressie en enzymactiviteit van deze gemuteerde genen werden daarop geëvalueerd. Mutaties werden gevonden in drie gemeenschappelijke loci, gerelateerd aan twee promotoren van functionele genen en één metabole route. In overeenstemming met onze eerdere studie (hoofdstuk 2), vonden we mutaties, in de fluorideresistente stammen, in *mutp* en eveneens een verhoogde expressie van de fluoridetransporteiwitten in deze afgeleide stammen ten opzichte van hun moederstam. Dit onderstreept de rol van de fluoridetransporteiwitten bij de resistentie tegen fluoride. Er werden ook mutaties gevonden in de promotor *glpFp*, waarvan het stroomafwaarts gelegen gen (*glpF*) codeert voor een eiwit dat de opname van glycerol faciliteert. Een significant lagere expressie van *glpF* werd waargenomen in de twee fluorideresistente stammen in vergelijking met de wild-type stammen, wat de membraanpermeabiliteit en dus de influx van fluoride kan veranderen. De genen die coderen voor enolase (*eno*) en pyruvaatkinase (*pyk*), twee belangrijke

glycolytische enzymen, zijn ook gemuteerd in de fluorideresistente stammen. *S. mutans* C180-2FR heeft twee mutaties in *pyk*, terwijl UA159FR één mutatie in zowel *eno* als *pyk* heeft. We hebben vervolgens het effect van deze mutaties op de desbetreffende enzymactiviteit bepaald. In *S. mutans* C180-2FR werd pyruvaatkinase volledig gedeactiveerd door de aminozuursubstitutie Y419D. Enolase in *S. mutans* UA159FR werd minder geremd door fluoride in vergelijking met de wild-type stam UA159. De resultaten van hoofdstuk 4 gaven inzicht in mogelijke genen, gerelateerd aan het fluoride transport en de glycolyse, die betrokken kunnen zijn bij de ontwikkeling van fluorideresistentie.

Na het beschrijven van het genotype van fluorideresistente *S. mutans* stammen onderzochten we een belangrijk kenmerk van fluorideresistente stammen, namelijk de stresstolerantie, ofwel de "fitness", van de stammen. In **hoofdstuk 5** werd van zowel twee fluorideresistente stammen, namelijk *S. mutans* UF35 (beschreven in hoofdstuk 3) en UA159FR (beschreven in hoofdstuk 4), als hun wild-type stam UA159, de fitness bepaald. In plaats van planktonische culturen werden biofilms gebruikt, aangezien biofilms de bacteriële levensstijl in tandplak beter nabootsen. De fitness van de biofilms van de twee fluorideresistente stammen en hun wild-type werd onderzocht door ze bloot te stellen aan fluoride, chloorhexidine en letale pH (pH 3,0). Zowel *S. mutans* UF35 als UA159FR waren beter bestand tegen fluoride dan UA159. De *S. mutans* UA159FR biofilms waren beter bestand tegen chloorhexidine en lage pH dan de biofilms van UF35 en UA159. Daarnaast was de biomassa van UA159FR significant hoger dan van de andere twee stammen. Derhalve concludeerden wij dat de fitness van de fluorideresistente *S. mutans* stammen beter dan (UA159FR), of gelijk aan (UF35), de fitness van de wild-type stam was.

Naast de fitness is het vermogen om melkzuur te produceren, bijdragend aan de ontwikkeling van tandcariës, een belangrijk kenmerk van *S. mutans*. Vele studies rapporteerden dat, in aanwezigheid van fluoride, fluorideresistente *S. mutans* stammen meer melkzuur produceren dan fluoridegevoelige stammen, wat duidt op een hogere glycolytische activiteit in de fluorideresistente stammen. Dit kan het gevolg zijn van veranderingen in de regulering van de glucoseopname, wat ondersteund wordt door de experimenten in hoofdstuk 4. De mutatie die is geïdentificeerd in *pyk* en de veranderde activiteit van pyruvaatkinase in *S. mutans* C180-2FR kan leiden tot veranderingen in de intracellulaire concentratie van fosfoenolpyruvaat en

daardoor tot veranderingen in glucoseopname. In **hoofdstuk 6** vergeleken we de glucoseopnameactiviteit van een fluoridegevoelige met die van een fluorideresistente stam om de verschillen in zuurproductie in aanwezigheid van fluoride te bestuderen. De totale glucoseopnameactiviteit en de expressie van genen gerelateerd aan de glucoseopname, in afwezigheid en aanwezigheid van fluoride, werd gekwantificeerd voor *S. mutans* C180-2 en C180-2FR. Daarnaast werd de activiteit van het PEP-afhankelijke fosfotransferase systeem (PTS) in deze twee stammen bepaald. De glucoseopnameactiviteit in *S. mutans* C180-2FR werd aanzienlijk minder geremd door fluoride dan in C180-2. De aanwezigheid van fluoride leidde in C180-2 tot een duidelijke verhoging van de expressie van genen betrokken bij glucoseopname, terwijl geen verandering in de genexpressie werd waargenomen in C180-2FR. In afwezigheid van fluoride had C180-2 een significant hogere PEP-PTS activiteit dan C180-2FR. In aanwezigheid van fluoride vertonen de twee soortgelijke PEP-PTS activiteiten. De data uit dit hoofdstuk laten zien dat de glucoseopname in fluoridegevoelige en fluorideresistente stammen verschillend gereguleerd is. Het verschil in glucoseopname kan, zowel in afwezigheid, als in aanwezigheid van fluoride, worden gezien.

Samenvattend toont dit proefschrift aan dat fluorideresistente *S. mutans* stammen zowel fenotypische als genotypische veranderingen hebben ondergaan. Verscheidene genomische mutaties zijn geïdentificeerd in relatie tot fluorideresistentie. Een andere regulering van de fluoride-transport eiwitten kan een efficiënte en essentiële manier zijn voor *S. mutans* om resistent tegen fluoride te worden. Andere factoren, waaronder veranderingen in de glycolyse, kunnen ook bijdragen aan fluorideresistentie. Wanneer de bacteriën fluorideresistent zijn geworden, verandert hun glucoseopname, de fitness en uiteindelijk het vermogen om te overleven in de mondholte.

中文总结

氟化物作为最有效的防龋药物应用于临床已五十余年，口腔细菌在长时间暴露于高浓度氟化物后有可能发生对氟的耐受，即产生耐氟性。目前关于致龋性研究最多的细菌是变异链球菌，本论文将对一系列耐氟变形链球菌株的表型及基因型特点进行研究。

第二章研究了耐氟变形链球菌 C180-2FR 及其亲本菌株 C180-2 的特点。C180-2FR 在高氟环境中的生长能力显著高于 C180-2，两种菌株菌落形态表现出明显差异。对两个菌株进行全基因组鸟枪测序（WGS）及全基因组序列比对，以定位与耐氟性有关的基因。并利用 Sanger 测序法验证 C180-2FR 基因组中的单核苷酸多态性（SNP），对含有 SNP 的基因进行了基因表达的检测，以研究这些基因组变化的功能。基因组比对中发现 C180-2FR 中有 8 个 SNP，位于 5 个基因和 2 个基因间区域。基因表达的结果显示一个含有 SNP 的启动子下游 3 个基因在 C180-2FR 中较在 C180-2 中持续性上调，其中 2 个基因（*perA* 和 *perB*）编码氟转运体基因 *eriC*[F] 的同源基因。该研究表明 WGS 是一种发现耐氟变形链球菌基因组中变异的有效工具。研究为进一步探究耐氟机制提供了候选基因。

启动子 *mutp* 下游基因可编码编码氟转运体，第三章研究了 *mutp* 中的突变与 C180-2FR 耐氟性的关系。我们构建了一个含 *mutp* 突变的变形链球菌菌株 UA159 变异株，将其命名为 UF35，并对其在有氟环境下生长和产酸的能力进行检测。UF35 相较于 UA159 可在更高氟浓度下生长，同时可代谢产生更多乳酸。但耐酸反应（ATR）显示 UF35 相较野生株 UA159 对酸更敏感，这可能与氟转运体介导的消耗能量的“质子无效循环”有关。随后通过下游基因表达及报告菌株荧光量检测了 *mutp* 突变对启动子活性的影响。结果都表明变异后的 *mutp* 相较野生型 *mutp* 更加活跃。该研究表明 *mutp* 中的单核苷酸变异可提高启动子活性，并上调氟转运体的表达，由此增强变形链球菌的耐氟性。

以上研究证实了氟转运体基因调节和细菌耐氟性的关系，但它并不是细菌获得耐氟性的唯一方式。既往研究发现同一个亲本菌株能发展出具有不同程度耐氟性的子代菌株，表明耐氟性的发展过程中可能包括多个基因。为了识别其他与耐氟性相关的因素，在第四章中，我们分析了两个自然选择产生的耐氟菌株（UA159FR 和 C180-2FR）与他们的亲本菌株（UA159 和 C180-2）的基因组序列，识别了两个耐氟菌株共有的含突变染色体区域，对相应的基因表达和酶活性也进行检测。结果发现突变

位于 3 个共有区域，与两个功能基因启动子和一个代谢通路相关。与第二章所描述的研究一致，我们在两个耐氟菌株的 *mutp* 区域都发现了突变，并检测到氟转运体在两个耐氟菌株中较亲本菌株都明显高表达。这再次验证了氟转运体在耐氟性中的重要作用。除此之外，我们在 *glpFp* 发现突变，其下游基因 *glpF* 编码一个甘油摄取促进蛋白。两个耐氟菌株中 *glpF* 的表达明显低于亲本菌株，这可能导致细胞膜渗透性的变化并影响细胞对氟的摄入。编码烯醇酶（*eno*）和丙酮酸激酶（*pyk*）两个糖代谢关键酶的基因也在两个耐氟菌株中发生了突变。C180-2FR 在 *pyk* 中含有两个突变，UA159FR 则在 *eno* 和 *pyk* 中各有一个突变。酶活性检测发现 C180-2FR 的丙酮酸激酶由于氨基酸替换 Y419D 而完全失活，UA159FR 的烯醇酶相较 UA159 的烯醇酶被氟抑制的程度更小。本章结果为耐氟性机制的研究提供了新的候选基因，这些基因与氟转运和糖代谢有关。

在研究了耐氟变形链球菌的基因型特点后，我们进一步检测了耐氟菌株的另一重要特性，即对环境压力的耐受性，又称细菌的适应性（fitness）。在第五章中我们研究了 UF35、UA159FR 以及它们的野生菌株 UA159 在生物膜状态下的适应性。将三种细菌生物膜暴露于氟、氯己定和酸（pH3.0）的环境压力中，结果表明 UF35 和 UA159FR 相较野生菌株 UA159 都表现出对氟更强的耐受性，UA159FR 生物膜的量和对氯己定和强酸的耐受性均显著高于 UA159 和 UF35。我们由此总结耐氟变形链球菌的适应性等于（UF35）或强于（UA159FR）野生菌株。

除了适应性之外，变形链球菌的产酸性也是其导致龋病发展的重要表型。多项研究表明耐氟变形链球菌在有氟环境下显示出更强的产酸能力，说明有氟环境中耐氟菌的糖代谢活力更强。由第四章的数据推断，这可能是葡萄糖摄取的调控发生改变导致的。C180-2FR 中发现的位于 *pyk* 的基因突变和丙酮酸激酶酶活性的改变可导致胞内磷酸烯醇式丙酮酸（PEP）浓度改变并由此调控葡萄糖摄取。在第六章中，我们量化分析了变形链球菌 C180-2 和 C180-2FR 的无氟和有氟环境下糖摄取总活性及糖摄取相关基因表达，同时检测了这两种细菌内 PEP 介导的磷酸转移酶系统（PTS）活性。结果发现有氟环境下 C180-2FR 糖摄取受到的抑制较 C180-2 明显更小。氟的存在诱发了 C180-2 内糖摄取相关基因的显著高表达，而在 C180-2FR 内并没有基因调控的发生。在无氟环境中，C180-2 的 PEP-PTS 活性显著高于 C180-2FR，在有氟环境中，两个菌种 PEP-PTS 活

性无明显差异。本章数据揭示了氟敏感和耐氟菌种的糖摄取被差异性调控，这种差异在无氟和有氟环境中皆存在。

综上所述，通过本研究我们筛选出了耐氟变形链球菌基因中多个与耐氟性相关的基因突变，证明了耐氟变形链球菌表型和基因型的改变。氟转运体的调控可能是变形链球菌获得耐氟性的一种关键而有效的方式。其他因素，如糖代谢的改变，也可能在耐氟机制中发挥一定作用。获得耐氟性后，细菌适应性和糖摄取均发生改变，这些表型变化可影响细菌在口腔中各种环境压力下的生存能力。

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List of publications and conferences

Pulications in this thesis

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Liao Y, Brandt BW, Zhang M, Li J, Crielaard W, van Loveren C, Deng DM. 2016. A Single nucleotide change in the promoter *mutp* enhances fluoride resistance of *Streptococcus mutans*. Antimicrob Agents Chemother 60(12):7509-7512.

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Other publications

Liang K, Gao Y, Li J, Liao Y, Xiao S, Lv H, He L, Cheng L, Zhou X, Li J. 2014. Effective dentinal tubule occlusion induced by polyhydroxy-terminated PAMAM dendrimer *in vitro*. RSC Advances 4(82):43496-503.

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Scientific conferences participation

ORCA 2015, Brussels, Belgium

Identification and functional analysis of genome mutations in a fluoride-resistant *Streptococcus mutans* strain. (Abstract and poster presentation)

OMIG 2016, Gregynog, Wales, United Kingdom
A single nucleotide change leads to enhanced fluoride resistance in *Streptococcus mutans*. (Oral presentation)

ORCA 2016, Athens, Greece
A single nucleotide change in *Streptococcus mutans* enhances fluoride resistance and alters virulence factors. (Abstract and poster presentation)

AMZA 2017, Amsterdam, the Netherlands
Fluoride resistance in *Streptococcus mutans*. (Oral presentation)

ORCA 2017, Oslo, Norway.
Identification of genomic mutations related to fluoride resistance in *Streptococcus mutans*. (Abstract)