Determination of arginine catabolism by salivary pellet

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ABSTRACT

To determine the formation of ammonium from arginine by oral bacteria residing in saliva and dental plaque, an arginolytic activity assay based on the work described by Nascimento et al. [2] was developed. Following the original methodology, insufficient ammonium production could be determined.

To improve the method for our research goal, the following modifications were made to the original protocols:

- The following changes were made to the arginine catabolism assay resulting in a 1000-fold increase in sensitivity: (i) the salivary pellet was washed and concentrated five times resulting in the removal of low density compounds interfering with the assay, (ii) the pH of the Tris–maleate buffer was increased from 6.0 to 7.5 resulting in a better conversion of arginine to ammonium and (iii) the incubation time was increased to 3 h to ensure that non-responders and salivary pellets low in cell numbers could yield detectable levels of ammonium.
- Removal of a centrifuge step from the protein determination resulted in a higher protein yield improving the accuracy of the assay.
- Changing from the use of the toxic, environmentally hazardous, mercury containing Nessler’s reagent to a colorimetric enzyme assay achieved a safer and greener determination of ammonium concentration.

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Methods

In short, the assessment of the arginolytic potential involved the following steps: (i) preparation of the salivary pellet to concentrate the oral bacteria present in the saliva; (ii) performing the arginolytic activity assay to assess the arginine catabolism; (iii) determination of the amount of ammonium produced from arginine; and (iv) determination of the protein weight of the salivary pellet. The final concentrations of ammonium were corrected per time unit and protein weight.

Preparation of the cell suspension

While optimizing the arginolytic activity assay, the pH of the Tris–maleate buffer was raised from pH 6.0 to pH 7.5. This was done as the arginolytic activity of a range of oral micro-organisms and the salivary pellet significantly produced more ammonium under pH 7.5 as compared to pH 6.0. The cell suspensions were prepared as follows:

1. Stimulated human saliva from various donors was thawed.
2. Four milliliter of each saliva sample was centrifuged for 10 min at 4000 × g at 4°C.
3. The salivary pellet was washed once with 800 ml 10 mM Tris–maleate buffer, pH 7.5 (Sigma) to remove low density salivary components.
4. The pellet was re-suspended in a final volume of 800 μl Tris–maleate buffer.

Arginolytic activity assay

In comparison to the original protocol the incubation time was lengthened to three hours. This was done to ensure that ammonium formation, in salivary pellets, low in cell amounts and saliva from non-responders, was still measurable.

To assess the arginolytic activity of the prepared cell suspensions, the following protocol was followed:

1. Of each cell suspension 237.5 μl was pipetted, in duplicate, into a non-skirted 96-well PCR plate (Corning) and pre-incubated at 37°C in a thermocycler (Eppendorf).
2. The remaining cell suspension was stored at −20°C for protein determination.
3. The arginolytic activity assay was started by adding 12.5 μl 1 M arginine to a final concentration of 50 mM (Sigma) to each cell suspension.
4. The plate with the cell suspensions was incubated at 37°C for 3 h.
5. Immediately after the addition of the arginine, and after the three-hour incubation period, 50 μl samples were taken, transferred to a PCR plate (VWR) and placed on ice for 5 min.
6. After cooling, the samples were heat inactivated for 5 min at 80°C, to stop all enzymatic reactions.
7. The PCR plate was centrifuged for 10 min at 1509 × g at 4°C.
8. The supernatants were transferred to a microtiter plate (Greiner), the plate was sealed and stored at −20°C until further analysis.

Ammonium determination

To determine the amount of ammonium produced from the catalysis of arginine, an assay was used as based on the method of Da Fonseca et al. [1].
Preparation of the reagents

1. The reaction buffer (TK-buffer) containing 0.5M triethanolamine and 15mM α-ketoglutaric acid (both Sigma), pH 8.0 was prepared. This buffer was stored at 4 °C for maximum of 4 weeks.
2. Fresh NADH stock solution was prepared by dissolving 40mg NADH (Roche) in 10ml TK-buffer.
3. To create a NADH work solution, the NADH stock solution was further diluted (10 times) in TK-buffer. This NADH work solution was stored on ice until further use.
4. The GIDH stock solution was prepared by adding 3.6ml MilliQ water to 3000U glutamate dehydrogenase (Roche). This stock solution was stored at 4 °C.
5. Prior to the assay, a GIDH work solution was prepared by diluting the GIDH stock solution, 10 times in MilliQ water. The GIDH work solution was stored on ice until further use.
6. A 4mM ammonium stock was prepared by diluting a 40mM ammonium sulfate (NH₄)₂SO₄ (Sigma), 20 times in TK-buffer.
7. Ammonium standards were prepared in the range of 0–2.8mM, with 0.4mM intervals.

Ammonium assay

1. A 96-well microtiter plate (Greiner) was filled with 120µl MilliQ water per well.
2. 65µl of NADH work solution was added to each well.
3. 10µl samples (obtained from the arginolytic activity assay) were added in duplicate, to the appropriate wells.
4. 10µl of the eight standards was added in duplicate to the appropriate wells.
5. The plate was mixed for 5min on a microtiter plate shaker.
6. The absorbance A₁ (λ =340nm) was read.
7. The enzymatic reaction was started by adding 20µl GIDH working solution to each well.
8. The plate was mixed for 30s on a microtiter plate shaker.
9. The plate was incubated for 20min at 20–25 °C.
10. The absorbance A₂ (λ =340nm) was read.

The ΔA (A₁ – A₂) was calculated and the values extrapolated against the ammonium standards. The calibration curve for the ammonium assay was linear up to 3.5mM ammonium. The lower detection limit was defined as the average absorbance of the lowest concentration measured plus three times the standard deviation. The upper detection limit was defined as the average absorbance of the highest concentration measured minus three times the standard deviation. Fig. 1 shows an example of the reproducibility and accuracy of the standard curve including the upper and lower detection limit.

The obtained ammonium values were corrected for time and protein mass. To illustrate the sensitivity of the method, the Graphical Abstract shows the arginolytic activity of saliva from nine different donors, expressed as the amount of ammonium (nM) produced per minute per µg protein.

Protein determination of the salivary pellet

The protein concentration of the cell suspensions used in the arginolytic activity assay was determined to normalize the ammonium formation by the salivary pellets with regards to differences in biomass.

In the original protocol [2], a centrifuge step had to be performed after the bead beating steps. Unfortunately, by centrifuging the samples, the cell walls were pelleted on top of the glass beads (see Graphical Abstract). As a result, less protein is present in the supernatant. This subsequently leads to an underestimation of the protein concentration, as the supernatant is used for the protein determination according to Bradford. Hence this centrifugation step was omitted from the method.

To perform the modified protein determination the following steps were taken:

1. A 2ml 96-deepwell plate (Greiner) was filled with 200µl MilliQ water washed Ø=0.1mm glass beads (Biospec products) per well.
2. The samples from stored at step 2 of the arginolytic activity assay were thawed.
3. 100 μl of each sample was pipetted in duplicate, into the deepwell plate.
4. The plate was sealed with a silicone mat.
5. The plate was subjected to two bead-beat cycles comprising of 30s bead-beating, using a mini-
   Beadbeater 96 (Biospec products).
6. After each bead-beat step, the samples were cooled for 5 min on ice to prevent heating of the
   sample.
7. The deepwell plate was placed on a microtiter plate shaker (Labocat), and vortexed for 1 min.
8. The plate was taken off the shaker and the glass beads were left to settle for 5 min.
9. A 75 μl sample of the supernatant above the glass beads was taken.
10. The samples were transferred to a 96-well microtiter plate (Greiner).
11. The microtiter plate was placed on the microtiter plate shaker to center any residual glass beads.

Determine the protein concentration of the supernatant, in duplicate, according to the method of
Bradford [3].

12. Each well of a 96-well microtiter plate (Greiner) was filled with 185 μl MilliQ water and 50 μl Bio-
    Rad Protein assay reagent.
13. Standards were prepared using bovine serum albumin (Sigma).
14. 15 μl of supernatant from step 11 was added to the reaction mixtures.
15. The samples were mixed vigorously by pipetting and 100 μl of the mixtures was transferred to a
    new 96-well microtiter plate.
16. The absorbance (λ = 595 nm) was determined and the protein concentration calculated.

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References


The Na$_v$ channel bench series: Plasmid preparation

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ABSTRACT

Research involving recombinant voltage-gated sodium (Na$_v$) channels has unique challenges. Multiple factors contribute, but undoubtedly at the top of the list is these channels' DNA instability. Once introduced into bacterial hosts, Na$_v$ channel plasmid DNA will almost invariably emerge mutagenized and unusable, unless special conditions are adopted. This is particularly true for Na$_v$1.1 (gene name SCN1A), Na$_v$1.2 (SCN2A), and Na$_v$1.6 (SCN8A), but less so for Na$_v$1.4 (SCN4A) and Na$_v$1.5 (SCN5A) while other Na$_v$ channel isoforms such as Na$_v$1.7 (SCN9A) lie in between. The following recommendations for Na$_v$ plasmid DNA amplification and preparation address this problem. Three points are essential:

- Bacterial propagation using Stbl2 cells at or below 30°C.
- Bias toward slow-growing, small bacterial colonies.
- Comprehensive sequencing of the entire Na$_v$ channel coding region.

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Methods

Step 1: transformation

Materials

- high quality starter Na_v plasmid DNA
- Max Efficiency® Stbl2 competent cells (Life Technologies)
- LB plates (with antibiotic at half-standard concentration)
- SOC or comparable outgrowth medium
- dedicated 30°C incubator and shaker

Note: This list includes only non-standard items. Generic components such as water baths and baffled flasks are assumed to be available.

Analyses of Na_v channel function begin with the construction or purchase of an expression vector (e.g., pCMV-Scrip, pCI-neo) containing a Na_v channel’s coding sequence. Alternative splicing is common among Na_v channels, creating different proteins from the same gene with distinct spatial and temporal expression patterns. Careful planning is therefore necessary to determine exactly which isoform and coding sequence are needed to address the questions of interest. With approximately 6 kb in length, Na_v channel coding sequences are long, making final insert-vector plasmid construct sizes in excess of 11 kb common. Regardless of its origin, be it a gift from a colleague or purchase from a commercial supplier, comprehensive sequence information ought to be part of the plasmid construct transaction. This will allow the end user to be fully informed when it comes to restriction analysis of the plasmid, which should always be the first step upon the DNA’s arrival. Simple electrophoresis of small aliquot of plasmid DNA digested with a reliable enzyme that produces a known banding pattern will show whether the plasmid is correct or not. This may seem trivial, but cases where providers less experienced with Na_v channel handling send corrupted DNA are not uncommon. If above mentioned restriction digest does not deliver the expected results (be selective here; minimal changes can be telltale signs of problems), digest with an alternative enzyme to confirm.

Once the restriction enzyme digestions delivered the appropriate restriction fingerprint, bacterial transformation can begin. We have had good success with chemical transformation as described below, but electroporation of suitable bacterial strains may be a viable alternative. Contrary to standard transformations, competent cell choice is critical when comes to Na_v channels. Among the cell lines known to be susceptible to produce problems are JM101, DH5α, One Shot Top10®, to name but a few. Transforming these cells according to the manufacturer’s instructions has a high chance of producing a negative outcome, although in select cases success appears to be possible (JM109, unpublished data). In our hands, by far the most reliable and successful is the Stbl2 bacterial strain, whose genotype favors cloning of problematic plasmid DNA. Although somewhat costly, it is possible to refreeze them at –80°C in 25-µl ready-to-use aliquots using immediate immersion in ethanol/dry ice without losing too much efficiency, should a more economical solution be necessary.

Once the transformation is completed, the bacteria are propagated on LB plates with an appropriate antibiotic, at approximately half-standard concentration (e.g., 50 vs. 100 µg/ml ampicillin), at 27–30°C. Because this temperature is lower than the 37°C incubation used in standard procedures, using a lower concentration of antibiotic will allow faster bacterial growth without compromising the selection of resistant clones. It is very important at this point to emphasize the use of a dedicated and appropriately labeled 30°C incubator. Any temperature elevation of Stbl2 cells above 30°C will inevitably corrupt Na_v channel DNA through host-mediated mutation (personal observation). At this temperature, the
growth of bacteria harboring \( \text{Na}_v \) channel DNA is very slow, and incubations of 3–5 days are often necessary. At no time during this period can the temperature exceed 30 °C. Appropriate signage ought to be attached to the incubators to ward off secondary users who may briefly change the incubator temperature to the standard 37 °C unbeknownst to the \( \text{Na}_v \) channel researcher.

**Procedure**

- ice-chill 2 µl (20 ng) of plasmid DNA in the bottom of a Falcon tube
- thaw a 25–µl aliquot of Stbl2 cells on ice for 5 min, then pipette over the chilled DNA and mix with gentle pipette tip agitation
- incubate on ice for 25 min, then heat shock in a 42 °C water bath for 40s and immediately return to ice for 2 min
- add 400 µl of room-temperature SOC and shake in a pre-warmed incubator at 30 °C for 90 min
- plate 50 and 250 µl of the cells on two separate, pre-warmed LB plates with antibiotic at half-standard concentration
- following a 10-min right-side up absorption at room temperature, place the plates upside-down in the dedicated 30 °C incubator
- allow to incubate for several days until two distinct populations of colonies have emerged: large fast-growers and tiny slow-growers

**Step 2: bacterial growth**

**Materials**

- dedicated 30 °C incubator/shaker
- 11 LB broth (selective antibiotic at half-standard concentration)

*Note:* As in Step 1, this list includes only non-standard items. Also required are commercial plasmid prep kits (mini as well as maxi), gel electrophoresis instrumentation, and associated consumables.

As mentioned earlier, the success of the transformation will not be known for several days. Although colonies might be visible the morning after the first overnight incubation, these fast growing colonies very likely carry corrupted plasmid DNA and should be avoided. In the cases that we have examined, the extent of the associated genetic alterations in fast growing colonies varied greatly, ranging from point mutations to large-scale rearrangements. The best approach is to wait for 2–3 days before the plates are scanned for colony growth, in which time, a second, slow-growing population of exceedingly small colonies will become discernible. To make colony spiking easier, incubation at 30 °C or room temperature may continue for up to 5 days. Care should be taken when ampicillin is used as the selective antibiotic. Rapidly growing resistant colonies will secrete beta-lactamase that can allow growth of non-resistant cells, which will appear as small, slowly-growing satellites surrounding fast-growing colonies. Bias toward slowly-growing, well-separated colonies helps avoid this issue. An alternative approach is to use an antibiotic such as for tetracycline for which resistance is only effective in the cells that express the resistant gene.

We recommend spiking 10–20 colonies to generate standard LB mini-cultures; the concentration of the selective antibiotic should be identical to that of the previously used plate. Shaking proceeds at standard speeds in standard round-bottom tubes. Maintenance of the 30 °C rule is crucial, which translates into 2–3 days of growth to reach the desired density, although shorter incubation times have been observed. We have experimented with richer media such as terrific broth, but the results were mixed. Terrific broth does accelerate bacterial growth, but it is also more prone to overgrow, which creates downstream problems in plasmid isolation if the growth is not stopped in time.

Once the cultures have reached the appropriate turbidity, they are processed with any standard mini plasmid prep kit since no one commercial kit is advantageous over another. However, it is important to use the maximal amount of cell material allowable per manufacturer's instructions; we...
commonly process 1.5 ml of culture. The eluted DNA is subjected to the same restriction analysis 
employed upon arrival of the original DNA. For this digest, simple mixing of the eluate with restriction 
buffer and enzyme (without addition of water) should yield the best results, as the DNA concentration 
is likely very low. Alternatively, a smaller elution volume may be used to achieve more concentrated 
DNA samples.

Provided the mini culture analysis generated the expected restriction pattern, a maxi culture can be 
started. We commonly increase the manufacturer’s recommended culture volume by a factor of 2 or 3 
for the culture setup. In other words, if a 200-ml culture is suggested, we usually grow three baffled 
flasks of 200 ml LB with the aforementioned half-standard antibiotic concentration. Inoculation is 
done at 1:1000 by adding 200 μl of the original mini-culture (kept at 4 °C) per flask. Best practice is to 
process the mini-preps and inoculate the maxi-cultures on the same day.

Contrary to all previous incubations, the maxi cultures, while also grown at 30 °C, usually grow 
quicker and reach sufficient turbidity after only an overnight shake. One can now process the cultures 
according to the manufacturer’s recommendations. We do recommend individual processing of each 
maxi culture to avoid clogging of the filtration columns after alkaline lysis. However, the DNA from all 
three setups can be combined in the final step by serial passage of the eluate from one column to the 
next. Alternatively, one could simply combine the three eluates and reduce their volume in a speedvac, 
granted that water was used in the elution step.

**Procedure**

- following several days of growth, inspect the LB plates and identify suitable slow-growing, small 
  colonies
- using autoclaved toothpicks, inoculate several 3-ml LB mini cultures (half-standard antibiotic 
  concentration)
- shake at 30 °C for 2–3 days until the cultures turn turbid
- process 1.5 ml of mini cultures according to the instructions of the available mini prep kit using 
  water in the final elution step; store the remainder of the mini culture at 4 °C
- using only restriction buffer, enzyme, and eluate, set up 20-μl digests and separate with standard gel 
  electrophoresis
- identify a setup with the expected restriction fingerprint and 1:1000 inoculate two to three 200-ml 
  maxi cultures (LB, half-standard antibiotic concentration) with 200 μl of mini culture
- shake at 30 °C over night
- process each 200-ml culture separately according to the maxi prep kit manufacturer instructions; 
  only the final TE buffer elution step is customized: combine the DNA yield by sending the eluate 
  from the first silica column through the second (and the third, if three maxi cultures were 
  processed); intermittent volume adjustment of the eluate with additional TE buffer may be 
  necessary

**Step 3: DNA sequencing**

Once the maxi prep DNA has been obtained, standard spectrophotometric analysis will help 
determine the DNA concentration, and restriction fingerprinting as described earlier will confirm the 
isolated DNA’s identity. Despite all optimization efforts, the DNA yield may be low, which may 
interfere with DNA sequencing. Service facilities frequently request plasmid concentrations in excess 
of 1 μg/μl for double-stranded DNA larger than 10 kb. Therefore, the initial elution volume can be kept 
small to achieve higher concentration of working DNA, which can then be diluted to the desired level if 
needed. Moreover, it is our experience that close communications with the sequencing facility staff 
can also help address these issues. With additional care and informed personnel, we have not had any 
problems sequencing our Na+ channel plasmids, even at concentrations as low as 100 ng/μl.

Full-length sequencing encompassing the entire Na+ channel coding region is essential. With today’s DNA 
sequencing technology, primer walking with 600–800 bp intervals ought to generate reliable results.

We additionally process our chromatograph data using Nucleics’ online PeakTrace application, which 
aids in clearing base-calling ambiguities. All sequencing data is routinely aligned to our reference

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sequence (preferably an NM RefSeq or LRG record – hyperlinks provided in Additional information section) where an experienced reviewer visually confirms all base calls. Questionable areas ought to be re-sequenced and, if necessary, primed in both directions. We provide a list of sequencing primers in the online material of this article. Once the Na_\text{v} channel DNA has been certified in this fashion, it can be used for transfection and/or mutagenesis for downstream analysis of the channel function.

**Additional information**

**Background**

Voltage-gated sodium (Na_\text{v}) channels control excitation in brain, heart, and muscle. Deviations from their tightly defined functional characteristics, for example through genetic alterations, lead to heritable pathology that reflects the spatial and temporal expression pattern of the affected Na_\text{v} channel gene ([1–7], reviewed in [13]). Pharmacological control of the clinical symptoms in these so-called Na_\text{v} channelopathies (e.g., epilepsy, cardiac arrhythmia, muscle cramps, pain) is often hampered by treatment-emergent adverse effects due to structural and functional similarities among the Na_\text{v} channels that make isoform-specific targeting difficult. Improved intervention may be possible with patient-tailored therapy, where the drug action counters the defect of the faulty Na_\text{v} isofrom without affecting other Na_\text{v} channel function. Such drug development efforts are challenged by the unique requirements that come with recombinant DNA work involving Na_\text{v} channel coding sequences, because Na_\text{v} channels are notoriously difficult subjects when it comes to bacterial plasmid amplification, mutagenesis, and stable cell line development. The basis for this behavior is unknown, and no study researching the same has been published. One may speculate that the combination of exceptionally large coding sequences with internal homologous repeats is involved. There is evidence that Na_\text{v} channel DNA in itself is toxic for the bacterial host, since random mutation events are not limited to full-length Na_\text{v} channel coding sequences, but they also occur during bacterial propagation involving Na_\text{v} channel DNA fragments (personal observation).

**Useful links**

- PeakTrace software – www.nucleics.com/peaktrace
- LRG: Locus Reference Genomic – www.lrg-sequence.org

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**Appendix A. Supplementary data**

Further information on Na_\text{v}1.1 sequencing primers is provided as supplementary material. To view this information please refer to doi:10.1016/j.mex.2014.01.002.

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