Luciferase-Based Determination of ATP/NAD(H) Pools in a Marine (Environmental) Bacterium

Daniel Wünsch     Sabine Scheve     Arne Weiten     Kristin Kalvelage     Ralf Rabus

General and Molecular Microbiology, Institute for Chemistry and Biology of the Marine Environment (ICBM), Carl von Ossietzky University of Oldenburg, Oldenburg, Germany

Abstract
In all living organisms, adenosine triphosphate (ATP) and NAD(H) represent universal molecular currencies for energy and redox state, respectively, and are thus widely applicable molecular proxies for an organism’s viability and activity. To this end, corresponding luciferase-based assays in combination with a microplate reader were established with the marine model bacterium *Phaeobacter inhibens* DSM 17395 (*Escherichia coli* K12 served as reference). Grey multiwell plates best balanced sensitivity and crosstalk, and optimal incubation times were 5 min and 30 min for the ATP and NAD(H) assay, respectively, together allowing limits of detection of 0.042, 0.470 and 0.710 nM for ATP, NAD+, and NADH, respectively. Quenching of bacterial cell samples involved Tris-EDTA-DTAB and bicarbonate base-DTAB for ATP and NAD(H) assays, respectively. The ATP and NAD(H) yields determined for *P. inhibens* DSM 17395 at ¼ OD₉₀₀ were found to reside well within the range previously reported for *E. coli* and other bacteria, e.g., 3.28 µmol ATP (g cellsdry)⁻¹. Thus, the here described methods for luciferase-based determination of ATP/NAD(H) pools open a promising approach to investigate energy and redox states in marine (environmental) bacteria.

Introduction
ATP (adenosine triphosphate), the chemical energy currency, and NAD(H) (nicotinamide adenine dinucleotide), a central hydrogen-transferring cofactor in energy metabolism, are used by all living organisms and have been investigated for a long time, representing hallmarks of biochemistry. ATP was discovered in the late 1930s [e.g., Lohmann, 1929], its role in linking energy-consuming and -producing reactions recognised in the early 1950s [Lipmann, 1941], and its formation by ion-pumping ATP synthases elucidated thereafter [Skou and Esman, 1992; Boyer, 1993; Abrahams et al., 1994]. NADH was discovered at the beginning of the 20th century [Harden and Young, 1906], hydrid transfer during redox reactions indicated in the early 1950s [Lipmann, 1941], and its formation by ion-pumping ATP synthases elucidated thereafter [Skou and Esman, 1992; Boyer, 1993; Abrahams et al., 1994].
Luciferase-Based ATP/NAD-Assays

Microbial life, as reflected, e.g., by maintenance, adaptation, or growth, is tightly connected with the cellular energy (ATP) and redox (NAD(H)) states. Conversely, knowledge of intracellular ATP/NAD(H) content represents a quantifiable molecular proxy of viability and metabolic activity. This adds to our understanding of resource allocation between assimilation and dissimilation, and of growth efficiency during various conditions of growth [Bremer and Dennis, 1987; del Giorgio and Cole, 1998; Russel and Cook, 1995]. Thus, in combination with habitat-mimicking cultivation regimes, reliable determination of intracellular ATP/NAD(H) concentrations could provide a valuable estimate of viability and activity of a given study organism under natural settings.

In accord with the aforementioned general relevance of ATP and NAD(H), a wide range of analytical methods has been established for measuring concentrations of these key metabolites. The methodological approaches include (i) high-performance liquid chromatography (HPLC) [Ritov et al., 2004; Olafsson et al., 2017], (ii) spectrophotometry [Wagner and Scott, 1994; Brown et al., 2020], (iii) electrochemical biosensors/assays [Bergel et al., 1989; Deng et al., 2009] and (iv) in situ fluorescence detection [Baraghis et al., 2011; Wu et al., 2020]. Compared to these methods, luciferase-based assays combine high sensitivity and large dynamic range with ease of use and suitability for high-throughput applications by means of microplate readers equipped with the respective optics [e.g., Fan and Wood, 2007; Inglese et al., 2007; Necchi et al., 2017].

Luciferase (EC 1.13.12.7), member of the adenylate-forming enzyme superfamily [Gulick, 2009], oxidatively decarboxylates luciferin (LH₂) in an ATP-dependent manner to oxy luciferin (OxyLH₂) with concomitant emission of yellow-green light. The substrate LH₂ is composed of a benzothiazole and a thiazoline-carboxylic moiety, which are covalently linked via a C2′–C2 bond (Fig. 1a). The current view of the multi-step mechanism underlying the luciferase-based bioluminescence is schemed in Figure 1a and involves the following steps [Day et al., 2004; Marques and Esteves da Silva, 2009; Thorne et al., 2010; Hosseinkhani et al., 2011; Sundlov et al., 2012]: (i) in an initial nucleophilic displacement reaction, the carboxyl group at C4 of LH₂ attacks the α-phosphorous of Mg²⁺-ATP forming enzyme-bound luciferyl-adenylate (LH₂-AMP) and releasing Mg²⁺-PP₁ (Fig. 1a, top). (ii) Following base-catalysed H⁺-removal at C4 of LH₂-AMP, oxidation by molecular oxygen (O₂) yields the key LH₂-AMP hydroperoxide intermediate (di-oxetanone) and release of AMP (Fig. 1a, middle and bottom). (iii) Finally, decarboxylation at C4 of dioxetanone forms oxyluciferin with concomitant emission of light (Fig. 1a, bottom). The emission maximum (550–560 nm) apparently depends on the dissociation state of the hydroxyl group at C5 combined with the oxidation/dissociation state of the oxygen at C4.

To exploit luciferase-based assays also for the quantification of NAD(P)⁺/NAD(P)H, an upstream NAD(P)H-dependent diaphorase (EC 1.6.5.2) cycling reaction is employed [Zhou et al., 2014]. While the chemical composition of commercial assays is unknown (proprietary), the cycling reaction should proceed via the following principal steps. Diaphorase catalyses the NADH-dependent intramolecular lactonisation of a “trimethyl lock quinone” substrate yielding 6-hydroxyl cyanobenzothiazole next to the diamide and quinone moieties. Coupling with lactate-oxidising lactate dehydrogenase (EC 1.1.1.27) refurnishes NADH, thereby serving an amplifying function in the assay (Fig. 1b, top). Finally, excess of cysteine in the assay drives formation of luciferin from 6-hydroxyl cyanobenzothiazole under release of ammonium (Fig. 1b, bottom). Emission of light is achieved via the aforementioned luciferase-based reaction.

The aim of the present study was to establish and evaluate luciferase-based ATP/NAD(H) assays in multiwell plate format using a monochromator-based microplate reader for application with marine (environmental) bacteria. For this purpose, the aerobic heterotrophic Phaeobacter inhibens DSM 17395 was selected, since it is genome-sequenced [Thole et al., 2012], well-studied on the physiological and proteogenomic level in our laboratory [Drüppel et al., 2014; Trautwein et al., 2018; Wiegmann et al., 2014; Wünsch et al., 2019, 2020] and a member of the alphaproteobacterial Roseobacter group, which is widespread in pelagic oceanic water bodies [Buchan et al., 2005; Luo and Moran, 2014; Simon et al., 2017]. Facultative anaerobic, intestinal Escherichia coli K12 [Blattner et al., 1997; Hayashi et al., 2006; Keseler et al., 2017], belonging to the gammaproteobacterial family Enterobacteria-
Fig. 1. Biochemical principles of luciferase-catalysed bioluminescence. **a** Proposed multi-step mechanism of luciferase-catalysed reaction involving ATP-dependent activation of luciferin (LH₂) to LH₂-AMP followed by oxidative conversion to dioxetane and final decarboxylation to oxyluciferin with concomitant emission of light (adapted from Marques and Esteves da Silva [2009] and Thorne et al. [2010]); note that the first two reactions are reversible and that at C4 of oxyluciferin, a keto-enol-tautomery is assumed.

This reaction principle was applied in the assay for quantification of ATP. **b** Diaphorase-catalysed cycling reaction involves NADH-dependent reductive intramolecular lactonisation of a “trimethyl lock quinone” (e.g., quinone-trimethyl lock-C2-diamine-2-cyanobenzothiazole carbamate) followed by rapid luciferin formation (adapted from Zhou et al. [2014]). The latter then fuels luciferase-catalysed light emission (see above). This reaction principle was applied in the assay for quantification of NAD⁺/NADH.
Luciferase-Based ATP/NAD-Assays

Methods/Design

Strains, Media and General Cultivation Conditions
E. coli K12 MG1655 (DSM 18039) [Guyer et al., 1981] and P. inhibens DSM 17395; originally deposited as Phaeobacter galliciensis [Buddruhs et al., 2013] were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany).

Routine cultivation, substrate adaptation of bacterial strains and main growth experiments were performed as follows: (i) E. coli K12 in 250 mL defined mineral M9-medium [Sambrook and Russell, 2001] containing 6 mM glucose (1,000-ml Erlenmeyer flasks, 37°C, 100 rpm); (ii) P. inhibens DSM 17395 in 250 mL defined marine medium [Zech et al., 2009] containing 15 mM glucose (1,000-ml Erlenmeyer flasks, 28°C, 100 rpm). Prior to any of the experiments described below, each of the two strains was adapted to the cultivation conditions described above over five passages, starting from glycerol stocks in each case. Main cultures were then inoculated from actively growing pre-cultures. Organic substrates were provided from sterile stock solutions. Purity of the cultures was confirmed by microscopic examination (Axiostar; Zeiss AG, Göttingen, Germany). All chemicals used were of analytical grade.

Evaluation of Quenching Methods
E. coli cultures were used to determine the most suitable quenching method for the ATP assay (online suppl. Fig. S1; see www.karger.com/doi/10.1159/000522414 for all online suppl. material). Per tested centrifugation temperature, three replicate cultures (each derived from an independent glycerol stock) were used. Each individual culture was harvested at an optical density (OD) of ~0.2. Two aliquots à 1 mL were immediately shock-frozen in liquid N2. Ten aliquots à 1 mL were centrifuged (20,800 × g, 5 min, 14°C or room temperature), the obtained cell pellets resuspended in the various quenching buffers, followed by shock freezing in liquid N2; the supernatant from 1 replicate was retained and also shock-frozen in liquid N2. The retention time was at 80°C. The tested quenching buffers were: 1× PBS buffer; 6 M guanidine HCl in 1× PBS; 0.2 M NaOH base buffer with 1% (v/v) dodecyltrimethylammonium bromide (DTAB); bicarbonate base buffer with 1% (v/v) DTAB; and Tris (pH 7.75) with 2 mM EDTA and 1% (v/v) DTAB. The depletion of glucose during growth of P. inhibens DSM 17395 was determined by HPLC using an Ultimate3000 system (ThermoFisher, Germering, Bavaria, Germany) equipped with an RI-detector (Shodex RI-101; Showa Denko GmbH, München, Germany) and operated at 75°C. Separation was achieved on an Eurokat column (300 × 8 mm, 10-µm bead size; Knauer, Berlin, Germany), using 5 mM H2SO4 as eluent administered at 1.2 mL min⁻¹. The retention time was at 5.6 min and the dynamic range from 25 µM to 10 mM. Due to superposing background signals in the HPLC separation profile, the depletion of glucose during growth of E. coli K12 had to be determined by means of an enzymatic assay (Glucose Assay Kit ab65333; Abcam, Cambridge, UK) according to the manufacturer’s instructions, with the reaction conducted at 37°C and 400 rpm in the dark. Fluorescent recording (excitation: 535 nm; emission: 587 nm) was achieved by using a microplate reader (CLARIOstar® Plus; BMG Labtech, Ortenberg, Germany).

Luciferase-Based Assays
To examine the suitability of different types of multiwell plates, the following commercial products were tested: white (Cat. # 655074; Greiner, Kremsmünster, Austria), grey (Cat. # 6002350; PerkinElmer, Waltham, MA, USA), black (Cat. # 655077; Greiner). Generally, sterile pipet tips and gloves were used. Samples and respective standards were always measured in triplicates.

ATP Assays
The ATP assays for growth experiments were conducted using the BacTiter-Glo® Assay (Cat. # G8231; Promega, Fitchburg, WI, DOI: 10.1159/000522414

References

Adeolu et al., 2016; Buddruhs et al., 2013; Guyer et al., 1981; Sambrook and Russell, 2001; Zech et al., 2009.
NAD(H) Assays

The NAD(H) assays were conducted using the NAD/NADH-Glo™ Assay (Cat. # G9071; Promega), following the manufacturer’s instructions. Initially, NAD/NADH-Glo™ Buffer and NAD/NADH-Glo™ Substrate were thawed and equilibrated to room temperature. Then, the Luciferin Detection Reagent was reconstituted by adding the whole content of the NAD/NADH-Glo™ Buffer to the NAD/NADH-Glo™ Substrate and gently mixing by inverting the solution for ~1 min. This reagent is stable for 24 h at room temperature and might be stored longer at colder temperatures (see manufacturer’s technical bulletin). The quenched samples were thawed on ice, diluted in Tris (pH 7.75) with 2 mM EDTA (dilution buffer ATP; DB ATP) and equilibrated to room temperature, shortly before assaying. Then, 25 µL of the diluted samples and ATP standard series (0.1–1,000 nM ATP in Tris-EDTA; 100 mM ATP-stock, Cat. # R0441; Thermo Scientific™, Waltham, MA, USA) were administered to the multiwell plate, followed by adding 25 µL BacTiter-Glo™ Reagent to each used well and immediate mounting of the plate into the CLARIOstar® Plus reader (procedure and exemplary layout see online suppl. Fig. S3). Before recording of the luminescence signal at 545–550 nm, the plate was mixed in orbital shaker mode (10 s, 400 rpm) and incubated for 5 min at 25°C. For assessing plate types and quenching methods, the dilution buffer may vary, depending on the quenching buffer used (online suppl. Fig. S1).

Discussion/Conclusion

Establishment of Luciferase-Based Assays

The luciferase-based assays were established on the basis of the ATP assay and involved evaluation of the suitability of different multiwell plate types, length of incubation time, quenching, and calibration methods. In case of quenching experiments, cultures of E. coli K12 were used.

Multiwell Plate Types

To assess horizontal crosstalk between adjacent wells (online suppl. Fig. S5A), multiwell plates with different colouring and design were compared. (i) White multiwell plates are usually applied for luciferase-based assays, since this colouring enhances the luciferase signal. (ii) Black multiwell plates are used in particular for fluorescent assays, producing signal strengths higher than luciferase assays. Since particles in the well walls block well-to-well transmission, the inter-well crosstalk is minimised, resulting in a better signal-to-noise ratio. (iii) Grey half-area multiwell plates are characterised by a crosstalk-minimising design (reduced well volume, increased inter-well distance, black spiked white colouring). Independent of the multiwell plate type tested, crosstalk due to light bleeding from neighbouring wells towards the overlying optics is minimised by the perforated aperture spoon implemented in the multiwell plate reader used here (online suppl. Fig. S5A). This aperture is automatically positioned between the movable optical detector and the specific well to be analysed, thereby excluding the undesirable bleeding signals. For each of the above-mentioned multiwell plate samples were thawed on ice, mixed via vortexing, and 100-µL samples were pipetted into a new micro-reaction cup for each dinucleotide and its respective pretreatment. Samples intended for assaying NAD⁺ were acidified with 100 µL 0.4 M HCl, mixed and incubated for 15 min at 60°C. Samples destined for NADH assaying were incubated for 15 min at 60°C (already basic bicarbonate base buffer). After letting both sample types cool at room temperature for 10 min, NAD⁺ samples were neutralised with 100 µL 0.5 M Trizma®, while NADH samples were neutralised with 200 µL HCl/Trizma® solution (1:2 dilution of 0.4 M HCl and 0.5 M Trizma®). Both sample types were then diluted using a 1:3 bicarbonate base buffer-HCl/Trizma® solution (dilution buffer NADH; DB NADH). 25 µL of diluted sample and the respective standard series (10–400 nM NAD⁺/NADH) were then administered to a multiwell plate, 25 µL NAD/NADH-Glo™ Detection Reagent was added to each occupied well, and the plate was immediately mounted into the CLARIOstar® Plus reader (procedure and exemplary layout see online suppl. Fig. S4). The plate was then mixed on orbital shaker mode (10 s, 400 rpm) and incubated for 30 min before recording the luminescence signal.
types, two different approaches were used to quantify crosstalk as described in the following.

Initially, a simple plate layout (online suppl. Fig. S5B) was used to measure crosstalk against a blank background when applying high ATP concentrations in the luciferase-based assay. According to this given plate setup, the CLARIOstar® software calculates a plate-/method-specific factor to level out the occurring horizontal crosstalk. The determined crosstalk factors were 0.05353% for the white plates, 0.00026% for the black plates, and 0.01277% for the grey plates.

Then, an ATP concentration series was used (considering the specific crosstalk factors) to assess the interplay between crosstalk and sensitivity when neighbouring wells harbour markedly different ATP amounts (online suppl. Fig. S5C). The 7-point concentration series was adjusted to the different multiwell plate types: 0.01–1,000 nM ATP for white and grey plates versus 0.05–1,000 nM ATP for the black plates. While the overall ATP concentration-dependent course of luciferase signals (luminescence) was similar for all three multiwell plate types (online suppl. Fig. S5D), sensitivity decreased from white via grey to black plates. Furthermore, wells containing very low ATP concentrations and neighbouring wells with high ATP concentrations exhibited ~10-fold increased luminescence due to crosstalk (online suppl. Fig. S5E).

Taken together, these experiments indicate the following two points: first, the grey multiwell plates were found to provide the best compromise between sensitivity and crosstalk for the luciferase-based assays. An additional advantage of these plates is the reduced assay/sample volume. Second, the general setup of multiwell plates needs to avoid direct proximity between samples with markedly differing concentrations of ATP.

Incubation Time

To ensure the optimal incubation time prior to analysing the luciferase-based assays with the microplate reader, three time series with respective standards and buffer system (ATP in Tris-EDTA and NAD(H) in bicarbonate base-HCl/Trizma®) were conducted (online suppl. Fig. S6). To best cover for high and low concentrations, the incubation time for the ATP and NAD(H) assays was set to 5 and 30 min, respectively.

Quenching Methods

Culture samples reserved for luciferase-based assays have to be pre-treated by centrifugation and decanting of supernatants followed by quenching of the cells. This is essential, since (i) media components, especially high salt concentrations, might inhibit the luciferase reaction, resulting in lower sensitivity [Abushaban et al., 2017]; and (ii) immediate processing and analyses of samples concurrent with ongoing cultivations (~8 h to over 200 h) are rather impracticable. The suitability of different quenching methods for determination of ATP was evaluated using E. coli K12 cultures harvested at OD<sub>600</sub> ~0.2, involving centrifugation at either 14°C or room temperature (Table 1 and online suppl. Figs. S1 and S7). Culture samples were applied instantaneously (“fresh cells”) to the ATP assay or following shock freezing in liquid N<sub>2</sub> (“shock-frozen cells”), and culture supernatants served as references to elucidate potential analyte losses due to harvesting and quenching, respectively. The tested quenching methods comprised PBS, guanidine HCl, NaOH-DTAB, bicarbonate base-DTAB, and Tris-EDTA-DTAB [e.g., Hoffner et al., 1999; Squirrell et al., 2002; Pinu et al., 2017; Sobol et al., 2021], most of which contain the anionic surfactant DTAB [Sachin et al., 2018]. In case of the latter three buffers, additional experiments with a heating step were conducted, which, however, re-

---

**Table 1. ATP concentration depending on the quenching method applied prior to luciferase-based ATP assay**

| Quenching | ATP [nM]<sup>a</sup> |
|-----------|----------------------|
|           | Culture broth        |
| No        | Fresh cells<sup>b</sup> 2.575 ± 0.100 |
|           | Shock-frozen cells<sup>c</sup> 1.675 ± 0.107 |
| Harvest at 14°C<sup>d</sup> | RT<sup>d</sup> |
| Yes       | Culture supernatant 0.006 ± 0.001 |
| PBS       | 2.111 ± 0.147 2.475 ± 0.091 |
| 6 M guanidine HCl | bd  bd |
| NaOH-DTAB | 1.736 ± 0.394 2.438 ± 0.600 |
| NaOH-DTAB + heating | 1.746 ± 0.360 2.450 ± 0.531 |
| Bicarbonate base-DTAB | 2.243 ± 0.229 1.920 ± 0.404 |
| Bicarbonate base-DTAB + heating | 2.287 ± 0.242 1.940 ± 0.387 |
| Tris-EDTA-DTAB | 2.515 ± 0.362 2.143 ± 0.186 |
| Tris-EDTA-DTAB + heating | 2.169 ± 0.193 2.162 ± 0.059 |

<sup>a</sup> Intracellular ATP concentrations (except for culture supernatant) were calculated from Y<sub>ATP</sub>/X assuming that 70% of a standard E. coli cell consists of water as described by Neidhardt [1987].<sup>b</sup> Based on 4 replicates. <sup>c</sup> Based on 6 replicates. <sup>d</sup> Based on 3 replicates per harvest temperature and quenching method.
revealed that this measure had no effect on the determined ATP concentrations. Application of guanidine HCl did not allow to detect ATP at all. While the centrifugation temperature has significant albeit opposing effects with NaOH-DTAB and bicarbonate base-DTAB, nothing like that was observed with PBS and Tris-EDTA-DTAB. Analyses of the culture supernatant indicated that cells are apparently getting less leaky during centrifugation at 14°C as compared to room temperature. Thus, in summary, using Tris-EDTA-DTAB for quenching of ATP samples followed by centrifugation at 14°C were chosen as optimal conditions for all further experiments. In case of NAD(H) samples, the quenching method with bicarbonate base-DTAB was applied as described in the technical manual of the NAD/NADH-Glo™ Assay and cell harvesting was conducted at the above-mentioned centrifugation temperature (14°C). Note that the buffers for preparation of standards and for sample dilutions were devoid of DTAB, in order to reduce any interfering effect with the luciferase-based assays.

**Calibration**

In order to determine assay-specific parameters, 10-point calibrations were conducted for the ATP and the NAD(H) assays, applying the buffer conditions established in the above section Quenching Methods (Fig. 2). For calibration, an ATP standard series (0, 0.1, 0.125, 0.25, 0.5, 1, 10, 100, 500, and 1,000 nM ATP in Tris-EDTA) and NAD⁺/NADH standard series (0, 10, 12.5, 15, 20, 50, 75, 100, 200, and 400 nM NAD⁺ or NADH in bicarbonate base-HCl/Trizma®) were measured 16 times across two 96-well plates, applying the respective assay conditions. The limits of detection (LOD), identification (LOI) and quantification (LOQ) were calculated according to the method of blank determination [Magnarsson and Örnemark, 2014]. For the ATP assay, the LOD was 0.042 nM, the LOQ was 0.141 nM and the lin-

**Fig. 2.** Quantitative determination of ATP (a), NAD⁺ (b) and NADH (c) using Ultra-Glo™ luciferase-based assays. For the 10-point calibrations, the applied standards covered the following concentration range: 0–1,000 nM for ATP and 0–400 nM for NAD⁺ and NADH. For each standard concentration, 16 replicates (orange dots) were dispensed at even distances across two 96-well plates and measured at 545–550 nm. Black dots: mean of respective standard concentrations. Diagonal lines: dashed black, calibration curve; dashed blue, 95% confidence interval. The equation of the respective linear regression and the corresponding R² are indicated in each plot. The limits of detection (LOD), identification (LOI) and quantification (LOQ) are indicated with grey dashed lines in the zoom-ins.
Luciferase-Based ATP/NAD-Assays

Table 2. Yields and concentrations depending on type of normalization

| Organism   | ATP  | NAD⁺ | NADH |
|------------|------|------|------|
|            | Y_ATPᵇ | Y_ATP/c | Y_ATP/Prot | Y_NAD⁺ᵇ | Y_NAD⁺/cell | Y_NAD⁺/Prot | Y_NADHᵇ | Y_NADH/c | Y_NADH/Prot |
|            | [µmol ATP (g cells)⁻¹] | [µmol ATP (mg protein)⁻¹] | [µmol ATP (cell)⁻¹] | [µmol NAD⁺ (g cells)⁻¹] | [µmol NAD⁺ (cell)⁻¹] | [µmol NAD⁺ (mg protein)⁻¹] | [µmol NADH (g cells)⁻¹] | [µmol NADH (cell)⁻¹] | [µmol NADH (mg protein)⁻¹] |
| E. coli    | 4.68 ± 0.18 | 2.01E-12 ± 7.84E-14 | 2.06E-09 ± 0.078 | 1.55 ± 0.11 | 6.65E-13 ± 4.92E-14 | 0.663 ± 0.049 | 3.50 ± 0.04 | 9.96E-14 ± 1.60E-14 | 0.099 ± 0.016 | 0.52 ± 0.009 |
| P. inhibens| 3.28 ± 0.46 | 1.407 ± 0.198 | 5.94 ± 0.90 | 2.85 ± 0.15 | 1.222 ± nd | 0.39 ± nd | 0.03 ± nd | 0.014 ± 0.002 | 0.06 ± 0.004 |

E. coli

Yield ranged to at least 1,000 nM; for the NAD⁺ assay, the LOD was 0.470 nM, the LOQ was 1.580 nM and the linearity ranged to at least 400 nM; for the NADH assay, the LOD was 0.710 nM, the LOQ was 2.360 nM and the linearity ranged to at least 400 nM. The high R² (>0.99) depicted in each plot underlines the linearity and confidence of each calibration. The procedural standard deviations were 5.19 nM for ATP, 4.61 nM for NAD⁺, and 0.81 nM for NADH.

Application for E. coli K12 versus Marine P. inhibens DSM 17395

The established luciferase-based assays for ATP and NAD(H) were then applied to the standard bacterium E. coli K12 and the marine bacterium P. inhibens DSM 17395. The aim was to profile ATP/NAD(H) contents across growth stages (¼ OD max, ½ OD max, ¾ OD max and OD max) of batch cultures typically used for OMICS-based studies. Furthermore, these profiles should be correlated with growth stoichiometric parameters (OD, CDW, TCC, substrate consumption and biomass-specific growth rate) determined in the same growth experiments. Cultivation was performed under oxic conditions in shake flasks in defined mineral medium provided with glucose as single source of organic carbon and energy. The resultant data are compiled in Figure 3 and Table 2.

Escherichia coli K12

The growth behaviour of E. coli strains in batch cultivations with defined mineral media (e.g., M9) and glucose as sole source of carbon and energy is probably one of the most studied and best understood cultivation processes. Therefore, we used E. coli K12 as a reference for comparison with marine P. inhibens DSM 17395. Growth of E. coli K12 is characterised by exponential growth until transition into a stationary phase due to carbon limitation. Even though exponential growth is generally regarded as ideal, unhindered growth (µ max) can result in the well-known effect of acetate overflow metabolism, which can be avoided by reducing µ in fed-batch cultivations [e.g., Basan et al., 2015; Bernal et al., 2016; Enjalbert et al., 2017]. This overflow effect underlies a complex, multifactorial, not well-understood process involving transcriptional and posttranscriptional regulation, postranslational modification of proteins, etc. Additionally, high concentrations of primary electron donors (NADH), observed at high growth rates, were previously reported to reduce the flux through the TCA cycle by allosterically inhibiting both citrate synthase and isocitrate dehydrogenase leading to acetate accumulation [Yao et al., 2016]. In the present study, the growth behaviour of E. coli K12 was as outlined above, with exponential growth abruptly transiting into a stationary phase upon glucose depletion (Fig. 3a, top). ATP yields decreased linearly from ¼ OD max to ¾ OD max, even though cells were still growing exponentially, albeit at a somewhat reduced rate at ¾ OD max (Fig. 3a, middle). One may speculate that this decline reflects inefficient and incomplete substrate catabolism as well as complex adaptations for transiting into the stationary phase [Yoshida et al., 2018]. This is accompanied by slightly rising NAD⁺ yields and sinking intracellular NADH levels (Fig. 3a, bottom). One explanation might be the aforementioned overflow metabolism, which has recently been attributed to higher cellular demand of NAD⁺ relative to ATP [Luengo et al., 2021].
Phaeobacter inhibens DSM 17395

The growth behaviour of *P. inhibens* DSM 17395 in standard sea water medium supplied with glucose is characterised by a short exponential growth phase followed by a long linear growth phase (Fig. 3b, top and middle). Growth is possibly limited due to N-limitation and inhibited by produced TDA, which dissipates the proton motive force [Trautwein et al., 2016, 2018; Wilson et al., 2016]. Additionally, the property of *P. inhibens* DSM 17395 to form aggregates [e.g., Drüppel et al., 2014] may increase metabolic constraints on individual cells in their centre, due to substrate deficiencies (e.g., C-source and oxygen). In accordance, the biomass-specific growth rate and the ATP yields declined in a somewhat exponential manner, implicating multiple possibly intertwined limitations and inhibitions. Likewise, the NAD⁺ yield declined in a similar manner (Fig. 3b, bottom), which might indicate a nitrogen limitation (among other factors) and constrain the profile recorded for *E. coli* K12. Such an interpretation would agree with the influence of intracellular nitrogen levels (2-oxoglutarate/glutamine ratio) on NAD⁺ production, involving the glutamine-dependent NAD⁺ synthetase (NadE), as proposed by Santos et al. [2020].

Comparison to Literature Data

To place the ATP and NAD(H) profiles determined in the present study in a larger context, the literature was searched for corresponding reports on bacteria. Intracellular concentrations of ATP or NAD(H) are typically
given in the units µM or mM, but when estimations for the biovolume of a cell are not available, cellular, biomass and/or protein yields are also eligible to compare the energy states between different strains and growth phases [e.g., Neuhard and Nygaard, 1987; Lasko and Wang, 1996; Tsapлина et al., 2007; Hammel et al., 2010]. For *E. coli*, a broad range of intracellular ATP concentrations has been reported, ranging from ~0.5 mM to ~9 mM, depending on media composition, growth substrates and growth phase [e.g., Neuhard and Nygaard, 1987; Lasko and Wang, 1996; Yaginuma et al., 2014; Deng et al., 2021]. The same holds true for NAD⁺ and NADH yields ranging from ~0.3 to ~4 µmol NAD⁺ (g cells[dry]⁻¹) or from ~0.03 to ~1.25 µmol NADH (g cells[dry]⁻¹) [London and Knight, 1966; Sanchez et al., 2005; Vemuri et al., 2006]. London and Knight [1966] also report an extremely wide range of NAD⁺ yields in various bacterial strains upon growth under anoxic versus oxic conditions, ranging from 0.19 µmol NAD⁺ (g cells[dry]⁻¹) in *Pseudomonas fluorescens* to 10.59 µmol NAD⁺ (g cells[dry]⁻¹) in *Streptococcus faecalis*. The present study revealed ATP and NAD(H) yields at ¼ OD max (per g cells[dry] or mg protein) and their concentrations (Table 2) to fall well into the ranges of the aforementioned literature data, e.g., 4.68 and 3.28 µmol ATP (g cells[dry]⁻¹) for *E. coli* K12 and *P. inhibens* DSM 17395, respectively. Notably, measured NADH values for *P. inhibens* DSM 17395 were quite low and near the detection limit, in contrast to *E. coli* K12.

**References**

Abrahams JP, Leslie AGW, Lutter R, Walker JE. Structure at 2.8 Å resolution of F₁·ATPase from bovine heart mitochondria. *Nature*. 1994 Aug;370(6491):621–8.

Abushaban A, Mangal MN, Salinas-Rodriguez SG, Nnebuo C, Mondal S, Goueli SA, et al. Direct measurement of ATP in seawater and application of ATP to monitor bacterial growth potential in SWRO pretreatment systems. *Desalin Water Treat*. 2017 Dec;99:91–101.

Adeolu M, Alnajar S, Naushad S, Gupta RS. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacteriales ord. nov. divided into the families Enterobacteriaceae, Erwinicaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. *Int J Syst Evol Microbiol*. 2016 Dec;66(12):5575–99.

Baraghis E, Devor A, Fang Q, Srinivasan VJ, Wu W, Lesage F, et al. Two-photon microscopy of cortical NADH fluorescence intensity changes: correcting contamination from the hemo-

dynamic response. *J Biomed Optics*. 2011 Oct;16(10):106003.

Basan M, Hui S, Okano H, Zhang Z, Shen Y, Williams JR, et al. Overflow metabolism in *Escherichia coli* results from efficient protomer allocation. *Nature*. 2015 Dec;528(7580):99–104.

Bergel A, Soupe J, Comtat M. Enzymatic amplification for spectrophotometric and electrochemical assays of NAD⁺ and NADH. *Anal Biochem*. 1989 Jun;179(2):382–8.

Bernal V, Castaño-Cerezo S, Cánovas M. Acetate metabolism regulation in *Escherichia coli*: carbon overflow, pathogenicity, and beyond. *Appl Microbiol Biotechnol*. 2016 Nov;100(21):8985–9001.

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, et al. The complete genome sequence of *Escherichia coli* K-12. *Science*. 1997 Sep;277(5331):1453–62.

Böyer PD. The binding change mechanism for ATP synthase – some probabilities and possibilities. *Biochim Biophys Acta*. 1993 Jan;1140(3):215–50.

Bremer H, Dennis PP. Modulation of chemical composition and other parameters of the cell at different exponential growth rates. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE, editors. *Escherichia coli and Salmonella typhimurium: cellular and molecular biology*. Washington, DC: ASM Press; 1987. Vol. 2. p. 1527–42.

Brown AS, Calcott MJ, Collins VM, Owen JG, Ackerley DF. The indigoldine synthetase BpsA provides a colorimetric ATP assay that can be adapted to quantify the substrate preferences of other NRPS enzymes. *Biotechnol Lett*. 2020 Dec;42(12):2665–71.

Buchan A, González JM, Moran MA. Overview of the marine *Roseobacter* lineage. *Appl Environ Microbiol*. 2005 Oct;71(10):5665–77.

Buddruhs N, Pradella S, Gökem M, Päuker O, Pukall R, Spierl C, et al. Molecular and phenotypic analyses reveal the non-identity of the *Phaeobacter gallaeciensis* type strain deposits CIP 105216² and DSM 17395. *Int J Syst Evol Microbiol*. 2013 Nov;63(11):4340–9.

Microbiol Physiol 2022;32:122–133

DOI: 10.1159/000522414

Luciferase-Based ATP/NAD-Assays

Statement of Ethics

Ethical approval was not required since the study involved environmental bacteria.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was supported by the Deutsche Forschungsgemeinschaft (award SFB TRR 51).

Author Contributions

D.W. and R.R. conceived the study; A.W., D.W., K.K. and S.S. conducted the cultivation experiments; D.W. and S.S. established and conducted luciferase-based assays; S.S. determined substrate depletion profiles; D.W. analysed the data; R.R. wrote the manuscript with contributions from D.W. All authors have agreed to the final version of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material. Further enquiries can be directed to the corresponding author.

DOI: 10.1159/000522414
Chenuault HK, Whitesides GM. Regeneration of nicotinamide cofactors for use in organic synthesis. Appl Biochem Biotechnol. 1987 Mar; 14(2):147–97.

Day JC, Tisi LC, Bailey MJ. Evolution of beetle bioluminescence: the origin of beetle luciferin. Luminescence. 2004 Jan/Feb;19(1):8–20.

del Giorgio PA, Cole JJ. Bacterial growth efficiency in natural aquatic systems. Annu Rev Ecol Syst. 1998;29(1):503–41.

Deng C, Chen J, Nie L, Zie Y, Yao S. Sensitive bifunctional aptamer-based electrochemical biosensor for small molecules and protein. Anal Chem. 2009 Dec;81(24):9972–8.

Deng Y, Beahm DR, Ionov S, Sarapeshkar R. Measuring and modeling energy and power consumption in living microbial cells with a synthetic ATP reporter. BMC Biol. 2021 May; 19(1):101.

Drüppel K, Hensler M, Trautwein K, Kollmehl S, Wöhlbier L, Schmidt-Hohen K, et al. Pathways and substrate-specific regulation of amino acid degradation in Pseudomonas inhibens DSM 17395 (archetypate of the marine Roseobacter clade). Environ Microbiol. 2014 Jan; 16(1):218–38.

Enjalbert B, Millard P, Dinclaux M, Portais J-C, Létisse F. Acetate fluxes in Escherichia coli are determined by the thermodynamic control of the Pta-AckA pathway. sci. Rep. 2017 Feb; 7:42135.

Fan F, Wood KV. Bioluminescent assays for high-throughput screening. Assay Drug Dev Technol. 2007 Feb;5(1):127–36.

Friedkin M, Lehninger AL. Esterification of inorganic carbon compounds by mammalian liver. J Biol Chem. 1949 Apr; 178(2):611–44.

Gulick AM. Conformational dynamics in the acyl-CoA synthetases, adenylation domains of non-ribosomal peptide synthetases, and firefly luciferase. ACS Chem Biol. 2009 Oct; 4(10):811–27.

Guerer RS, Reed RR, Steitz JA, Low KB. Identification of a sex-factor-affinity site in E. coli as y. Cold Spring Harb Symp Quant Biol. 1981; 45:135–40.

Hammes F, Goldschmidt F, Vital M, Wang Y, Egli S. Measurement and interpretation of microbial adenosine triphosphate (ATP) in aquatic environments. Water Res. 2010 Jul;44(13): 3915–23.

Harden A, Young WJ. The alcoholic ferment of yeast-juice. Part II. The coferment of yeast-juice. Proc Royal Soc B Biol Sci. 1906 Oct; 78(526):369–75.

Hayashi K, Morooka N, Yamamoto Y, Fujita K, Isono K, Choi S, et al. Highly accurate genome sequences of Escherichia coli K-12 strains MG1655 and W3110. Mol Syst Biol. 2006 Feb; 2:2006.0007.

Hoffner S, Jimenez-Misas C, Lunding A. Improved extraction and assay of mycobacterial ATP for rapid drug susceptibility testing. Luminescence. 1999 Sep/Oct;14(5):255–61.

Hosseinikhani S. Molecular enigma of multicolor bioluminescence of firefly luciferase. Cell Mol Life Sci. 2011 Apr;68(7):1167–82.

Inglese J, Johnson RL, Simeonov A, Xia M, Zheng W, Austin CP, et al. High-throughput screening assays for the identification of chemical probes. Nat Chem Biol. 2007 Aug;3(8):466–79.

Keseler IM, Mackie A, Santos-Zavala A, Billingron T, Bonavides-Martinez C, Caspi R, et al. The EcoCyc database: reflecting new knowledge about Escherichia coli K-12. Nucleic Acids Res. 2017 Jan 45(1):D543–50.

Kühlbrandt W. Structure and mechanisms of F-type ATP synthases. Annu Rev Biochem. 2019 Jun;88:515–49.

Lasko DR, Wang DIC. On-line monitoring of intracellular ATP concentration in Escherichia coli fermentations. Biotechnol Bioeng. 1996 Nov;52(3):364–72.

Lippman F. Metabolic generation and utilization of phosphate bond energy. In: Norden FF, Werkman CH, editors. Advances in enzymology and related subjects. New York: Interscience Publishers; 1941. Vol. 1. p. 99–162.

Lohmann K. Über die Pyrophosphatfraktion im Muskel. Naturwissenschaften. 1929 Aug; 17(31):624–5.

London J, Knight M. Concentrations of nicotinamide nucleotide coenzymes in microorganisms. J Gen Microbiol. 1966 Aug;44(2):241–54.

Luengo A, Li Z, Gui DY, Sullivan LB, Zagorulya V, Austin CP, et al. High-throughput method to evaluate serum bactericidal activity using bacterial ATP measurement and related topics. 2nd ed. 2014. ISBN 978-91-87461-59-0. Available from: www.eurachem.org.

Marques SM, Esteves da Silva JCG. Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions. IUBMB Life. 2009 Jan;61(1):6–17.

Mitchell P. Coupling of phosphorylation to electron transfer and proton translocation in the respiratory chain. J Cell. 2002. Feb;81(4):691–707.

Minnich SF. Metabolic generation and utilization of phosphate bond energy. In: Norden FF, Werkman CH, editors. Advances in enzymology and related subjects. New York: Interscience Publishers; 1941. Vol. 1. p. 99–162.

Morgan DJ, O’Shea EK, Bateman A, Kelly P, Kinlan AB, et al. An overview of the EcoCyc database. BMC Genet. 2013 Nov;14:68.

Neuhard J, Nygaard P. Purines and pyrimidines. In: Neuhard FC, Ingraham JL, Low BK, Magasanik B, Schaechter M, Umbarger HE, editors. Escherichia coli and Salmonella typhimurium: cellular and molecular biology. 2nd ed. Vol 1. Part I: Metabolism and general physiology, Section B2 Biosynthesis and conversion of nucleotides, Chapter 29. Washington, DC: ASM Press; 1987. p. 445–73.

Olafsson S, Whittington D, Murray J, Regnier M, Moussavi-Harami F. Fast and sensitive HPLC-MS/MS method for direct quantification of intracellular deoxyribonucleoside tri- phosphates from tissue and cells. J Chromatogr B Analyt Technol Biomed Life Sci. 2017 Nov;1068-1069:90–7.

Passonneau JV, Lowry OH. Chapter I. En: Enzymatic analysis. A practical guide. Totowa: Humana Press; 1993. p. 13–14.

Pinu FR, Villas-Boas SG, Aggio R. Analysis of intracellular metabolites from microorganisms: quenching and extraction protocols. Metablistes. 2017 Oct;7(4):53.

Ritov VB, Menshikova EV, Kelley DE. High-performance liquid chromatography-based methods of enzymatic analysis: electron transport chain activity in mitochondria from human skeletal muscle. Anal Chem. 2004 Oct;76(1):27–38.

Russell JB, Cook GM. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiol Rev. 1995 Mar;59(1):48–62.

Sachin KM, Karpe S, Singh M, Bhattarai A. Physicochemical properties of dodecyltrimethylammonium bromide (DTAB) and sodium dodecyl sulphate (SDS) rich surfactants in aqueous medium, at T = 293.15, 298.15, and 303.15 K. Macromol Symp. 2018 Jun;379(1):170034.

Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.

Sanchez AM, Bennett GN, San K-Y. Effect of different levels of NADH availability on metabolic fluxes of Escherichia coli chemostat cultures in defined medium. J Biotechnol. 2005 Jun;111(4):395–405.

Santos ARS, Gerhardt ECM, Parize E, Pedrosa FO, Steffens MBR, Chubatsu LS, et al. NAD+ biosynthesis in bacteria is controlled by global carbon/nitrogen levels via PII signaling. J Biol Chem. 2020 May;295(18):6165–76.

Sazanov LA. The mechanism of coupling between electron transfer and proton translocation in respiratory complex I. J Bioenerg Biomembr. 2014 Aug;46(4):247–53.

Simon M, Scheurer C, Meier-Kolthoff JP, Brinkhoff T, Wagner-Döbler I, Ulbrich M, et al. Phylogenomics of Rhodobacteraceae reveals evolutionary adaptation to marine and non-marine habitats. ISME J. 2017 Jun;11(6):1483–93.

Skou JC, Esmann M. The Na,K-ATPase. J Bioenerg Biomembr. 1992 Jun;24(3):249–61.
Luciferase-Based ATP/NAD-Assays

Sobol M, Leippe D, Hooper K, Arduengo M, Vidugiriene J. Bioluminescent nicotinamide adenine dinucleotide detection assays Part II: Choosing the right product for your application. Updated, revised May 2021. Available from: https://www.promega.de/resources/pubhub/bioluminescence-nicotinamide-adenine-dinucleotide-detection-assays-part-ii/. Squirrel DJ, Price RL, Murphy MJ. Rapid and specific detection of bacteria using bioluminescence. Anal Chim Acta. 2002 Apr;457(1):109–14.

Steigmüller S, Turina P, Gräber P. The thermodynamic H+\text{ATP} ratios of the H+-\text{ATPsynthases} from chloroplasts and Escherichia coli. Proc Natl Acad Sci. 2008 Mar;105(10):3745–50.

Sundlov JA, Fontaine DM, Southworth TL, Branchini BR, Gulick AM. Crystal structure of firefly luciferase in a second catalytic conformation supports a domain alternation mechanism. Biochemistry. 2012 Aug;51(33):6493–5.

Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev. 1977 May;41(1):100–80.

Thole S, Kalhoefer D, Vogel S, Berger M, Engelhardt T, Liesegang H, et al. Pseudoalteromonas galacaeiensis genomes from globally opposite locations reveal high similarity of adaptation to surface life. ISME J. 2012 Dec;6(12):2229–44.

Thorne NJ, Inglese J, Auld DS. Illuminating the genetic code. Nat Rev Genet. 2007 Dec;8(12):926–37.

Tsaplina IA, Zhuravleva AE, Isaivalov AD, Zakharckh LM, Krasil’nikova EN, Bogdanova TI, et al. The dependence of intracellular ATP level on the nutrition mode of the acidophilic bacteria Sulfobacillus tolerotolerans and Acetobacillus tolerans. Microbiology. 2007 Dec;76(6):654–62.

Vemuri GN, Altman E, Sangurdekar DP, Khorodsky AB, Eiteman MA. Overflow metabolism in Escherichia coli during steady-state growth: transcriptional regulation and effect of the redox ratio. Appl Environ Microbiol. 2006 May;72(5):3653–61.

Wagner TC, Scott MD. Single extraction method for the spectrophotometric quantification of oxidized and reduced pyridine nucleotides in erythrocytes. Anal Biochem. 1994 Nov;222(2):417–26.

Warburg O, Christian W. Pyridine, der wasserstoff übertragende bestandteil von gärungsfermenten. Helv Chim Acta. 1936 Jan;19(1):E79–88.

Wiegmann K, Hensler M, Wöhlbrand L, Ulbrich M, Schomburg D, Rabus R. Carbohydrate metabolism in Pseudoalteromonas galacaeiensis DSM 17395, a member of the marine Roseobacter clade. Appl Environ Microbiol. 2014 Aug;80(15):4725–37.

Wilson MZ, Wang R, Gitai Z, Seyedsayamdost MR. Mode of action and resistance studies unveil new roles for tropodithietic acid as an anticancer agent and the γ-glutamyl cycle as a proton sink. Proc Natl Acad Sci U S A. 2016 Feb;113(6):1630–5.

Wu Z, Liu M, Liu Z, Tian Y. Real-time imaging and simultaneous quantification of mitochondrial H2O2 and ATP in neurons with a single two-photon fluorescence-lifetime-based probe. J Am Chem Soc. 2020 Apr;142(16):7532–41.

Wünsch D, Trautwein K, Scheve S, Hinrichs C, Feender S, Blasius B, et al. Amino acid and sugar catabolism in the marine bacterium Phaeobacter inhibens DSM 17395 from an energetic viewpoint. Appl Environ Microbiol. 2019 Dec;85(24):e02905–19.

Wünsch D, Strijkstra K, Wöhlbrand L, Freese HM, Scheve S, Hinrichs C, et al. Global response of Phaeobacter inhibens DSM 17395 to deletion of its 262-kb chromid encoding antibiotic synthesis. Microbial Physiol. 2020 Jul;30:9–24.

Yaginuma H, Kawai S, Tabata KV, Tomiyama K, Kakiwaka A, Komatsuzaki T, et al. Diversity in ATP concentrations in a single bacterial cell population revealed by quantitative single-cell imaging. Sci Rep. 2014 Oct;4:6522.

Yao R, Xiong D, Hu H, Wakayama M, Yu W, Zhang X, et al. Elucidation of the co-metabolism of glycerol and glucose in Escherichia coli by genetic engineering, transcription profiling, and 13C metabolic flux analysis. Biotechnol Biofuels. 2016 Aug;9(1):175.

Yoshida H, Shimada T, Ishihama A. Coordinated hibernation of transcriptional and translational apparatus during growth transition of Escherichia coli to stationary phase. mSystems. 2018 Jul;3(4):e00057–18.

Zech H, Thole S, Schreiber K, Kalbhöfer D, Vogel S, Brinkhoff T, et al. Growth phase-dependent global protein and metabolite profiles of Phaeobacter gallaeciensis strain DSM 17395, a member of the marine Roseobacter-clade. Proteomics. 2009 Jul;9(14):3677–97.

Zhou W, Leippe D, Duellman S, Sobol M, Vidugiriene J, O’Brian M, et al. Self-immolative bioluminescent quinone luciferins for NAD(P)H assays and reducing capacity-based cell viability assays. ChemBioChem. 2014 Mar;15(5):670–5.