

# [<sup>3</sup>H]Adenine is a suitable radioligand for the labeling of G protein-coupled adenine receptors but shows high affinity to bacterial contaminations in buffer solutions

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**Abstract** [<sup>3</sup>H]Adenine has previously been used to label the newly discovered G protein-coupled murine adenine receptors. Recent reports have questioned the suitability of [<sup>3</sup>H]adenine for adenine receptor binding studies because of curious results, e.g. high specific binding even in the absence of mammalian protein. In this study, we showed that specific [<sup>3</sup>H]adenine binding to various mammalian membrane preparations increased linearly with protein concentration. Furthermore, we found that Tris-buffer solutions typically used for radioligand binding studies (50 mM, pH 7.4) that have not been freshly prepared but stored at 4°C for some time may contain bacterial contaminations that exhibit high affinity binding for [<sup>3</sup>H]adenine. Specific binding is abolished by heating the contaminated buffer or filtering it through 0.2-µm filters. Three different, aerobic, gram-negative bacteria were isolated from a contaminated buffer solution and identified as *Achromobacter xylosoxidans*, *A. denitrificans*,

and *Acinetobacter lwoffii*. *A. xylosoxidans*, a common bacterium that can cause nosocomial infections, showed a particularly high affinity for [<sup>3</sup>H]adenine in the low nanomolar range. Structure–activity relationships revealed that hypoxanthine also bound with high affinity to *A. xylosoxidans*, whereas other nucleobases (uracil, xanthine) and nucleosides (adenosine, uridine) did not. The nature of the labeled site in bacteria is not known, but preliminary results indicate that it may be a high-affinity purine transporter. We conclude that [<sup>3</sup>H]adenine is a well-suitable radioligand for adenine receptor binding studies but that bacterial contamination of the employed buffer solutions must be avoided.

**Keywords** *Achromobacter* · *Acinetobacter* · Adenine receptor · Bacterial buffer contamination · G protein-coupled receptor · Nosocomial infection · Purine transporter · Purinergic receptor · Radioligand binding studies · [<sup>3</sup>H]adenine

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## Abbreviations

<i>A.d.</i>	<i>Achromobacter denitrificans</i>
<i>A.l.</i>	<i>Acinetobacter lwoffii</i>
<i>A.x.</i>	<i>Achromobacter xylosoxidans</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
CHO	Chinese hamster ovary
[ <sup>3</sup> H]CCPA	[ <sup>3</sup> H]2-Chloro-N <sup>6</sup> -cyclopentyladenosine
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EGTA	ethyleneglycoltetraacetic acid
<i>E. coli</i>	<i>Escherichia coli</i>
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
IUPHAR	International Union of Pharmacology
LB	Lennox broth

[ <sup>3</sup> H]MSX-2	[ <sup>3</sup> H]3-(3-hydroxypropyl)-7-methyl-8-( <i>m</i> -methoxystyryl)-1-propargylxanthine
O/F	oxidation/fermentation
OD	optical density
[ <sup>3</sup> H]PSB-0413	[ <sup>3</sup> H]2-propylthioadenosine-5'-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride
[ <sup>3</sup> H]PSB-11	[ <sup>3</sup> H]8-Ethyl-4-methyl-2-phenyl-(8 <i>R</i> )-4,5,7,8-tetrahydro-1 <i>H</i> -imidazo[2,1-]purin-5-one
spp.	species
Tris	Tris(hydroxymethyl)aminomethan

## Introduction

Purinergic receptors play an important role in transmembrane signaling [1]. Currently, two distinct families are officially recognized by the International Union of Pharmacology (IUPHAR), i.e. receptors for the purine nucleoside adenosine (P1 or adenosine receptors) and receptors for purine and/or pyrimidine nucleotides (P2 receptors) [2–4]. Whereas the P1 receptor family comprises four subtypes, A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub>, and A<sub>3</sub>, all of which are G protein-coupled receptors (GPCRs), the P2 family is further subdivided into two subfamilies, P2Y (GPCRs) and P2X (ligand-gated ion channels) [2–4]. In addition to the nucleoside (adenosine) and nucleotide receptors, a receptor for the nucleobase adenine has recently been discovered by a reverse pharmacological approach identifying adenine as the natural ligand for a rat orphan GPCR [5]. A mouse orthologue (mMrgA10) of the rat adenine receptor was subsequently identified by sequence comparison [5]. Very recently, a new adenine receptor has been cloned from mice showing 82% identity in its amino acid sequence to mMrgA10 and 76% to the rat adenine receptor, indicating that the new receptor is a distinct adenine receptor subtype (Genbank nucleotide sequence accession numbers: new mouse adenine receptor, DQ386867; mMrgA10, XM\_195647; rat adenine receptor, AJ311952) [6, submitted]. Both adenine receptors that have been pharmacologically characterized are coupled to the inhibition of adenylate cyclase by G<sub>i</sub> protein [5, 6]. So far, no human receptor for adenine has been identified, although initial clues for the possible existence of human adenine receptors have been found [7]. Adenine receptors are structurally unrelated to P1 and P2 receptors and therefore constitute a new family of purinergic receptors for which we proposed the designation P0 (“P zero”) receptors [8] based on the structural relationships of the physiological agonists, adenine (P0) representing a partial structure of adenosine (P1) and adenosine again being a partial structure of adenine nucleotides (P2), such as adenosine triphosphate (ATP) or adenosine diphosphate (ADP).

Radioligand binding studies are widely used to characterize GPCRs on the protein level [9]. Adenine, the natural ligand of adenine receptors, is commercially available in tritium-labeled form ([<sup>3</sup>H]adenine) and has been used to characterize recombinant rat adenine receptors expressed in Chinese hamster ovary (CHO) cells [5] as well as natively expressed rat adenine receptors in rat brain [7, 10] using membrane preparations. The radioligand has been shown to be stable under the incubation conditions [10]. Furthermore, mouse adenine receptors natively expressed in NG108-15 (neuroblastoma × glioma hybrid) cell membranes were labeled by [<sup>3</sup>H]adenine [7]. Recently, we successfully applied [<sup>3</sup>H]adenine binding to detect the mouse adenine receptor protein recombinantly expressed in Sf21 insect cell membranes, which constitute a null background because they do not endogenously express any high affinity binding site for adenine [6].

Two recent poster presentations reported on problems with [<sup>3</sup>H]adenine binding to adenine receptors. In one study using whole rat brain membrane preparations, a high-affinity binding site was detected (apparent K<sub>i</sub> 57.5 nM) [11]. However, a very high B<sub>max</sub> value was found (281 pmol/mg protein), and the binding was almost completely blocked by 10 μM of hypoxanthine and abolished in the absence of Mg<sup>2+</sup>, indicating that the detected binding site was not identical with the G protein-coupled rat adenine receptor [11]. In another study, high affinity binding of [<sup>3</sup>H]adenine was detected even in the absence of added protein, and the authors suggested that [<sup>3</sup>H]adenine bound in a highly specific manner to the glass fiber filters used in the filtration assays [12]. IC<sub>50</sub> values were determined for five adenine derivatives and three compounds [adenine (18 nM [12]; rat: 29.9 nM [7], 18 nM [5]), 7-ethyladenine (30 μM [12]; rat: 47.3 μM [7]), 8-bromoadenine (14 μM [12]; rat: 17.3 μM [7]) showed similar IC<sub>50</sub> values as those previously determined for the rat adenine receptor, but two compounds [5'-deoxyadenosine (725 μM [12]; rat: 0.823 μM [10]), 2-fluoroadenosine (19 μM [12]; rat: 0.62 μM [7]) had much lower affinities for the unknown binding site labeled in the absence of added protein [12] than for the rat adenine receptor as previously determined [7, 10], indicating that the unknown binding sites labeled in the absence of added rat tissue were very different. Based on their results, the IJzerman group had suggested to avoid the use of [<sup>3</sup>H]adenine as a radiolabeled probe for the adenine receptor due to its putative specific, high-affinity binding to glass fiber filters [12].

That report prompted us to carefully reanalyze radioligand binding data obtained with [<sup>3</sup>H]adenine in our laboratory with the goal to find out the reason for the problems encountered in other laboratories. As a matter of fact, we had occasionally observed unusually high counts in a few experiments, which could, however, be avoided by

repeating the experiments under carefully controlled experimental conditions, including the use of freshly prepared buffer solutions. We have now performed a systematic study clearly showing that [<sup>3</sup>H]adenine is a suitable radioligand for the labeling of adenine receptors in various cells and tissues if bacterial contaminations are excluded. Furthermore, we identified three common gram-negative aerobic bacteria that grow in cold Tris buffer and express high-affinity binding sites for [<sup>3</sup>H]adenine.

## Materials and methods

### Chemicals

[8-<sup>3</sup>H]Adenine (27 Ci/mmol) was obtained from Amersham Biosciences (Munich, Germany). Tris was obtained from Acros Organics (Leverkusen, Germany) and dimethyl sulfoxide (DMSO) was from Fluka (Switzerland). All other chemicals and reagents were obtained from Sigma unless otherwise noted.

### Cell culture

Human embryonic kidney (HEK) 293 and CHO-K1 cells were grown as monolayers at 37°C (5% CO<sub>2</sub>) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine.

### Membrane preparations

Frozen rat brains were obtained from Pel Freez (Rogers, AR, USA) and thawed at 4°C. Cortex and striata were dissected, and membrane fractions were prepared as previously described [7]. Membrane preparations from CHO-K1 cells, and HEK293 cells were prepared as described [13, 14]. Membrane preparations from bacteria were obtained after growing them on agar plates and subsequent amplification of single strains in Lennox broth (LB) medium over night. Membranes were then prepared in analogy to the procedures used for mammalian-cell membranes [7, 13]. Protein concentrations were determined according to the method of Lowry [15].

### [<sup>3</sup>H]Adenine binding assays

Adenine binding assays were carried out as previously described [7]; however, in the absence of Mg<sup>2+</sup> and ethyleneglycoltetraacetic acid (EGTA), unless otherwise noted, as Mg<sup>2+</sup> and EGTA were found to have no effect on determined K<sub>i</sub> values (data not shown). In brief, membrane preparations (50 µg of protein, unless otherwise

indicated) were incubated with 10 nM [<sup>3</sup>H]adenine in 50 mM Tris-HCl, pH 7.4 in a total volume of 200 µl. Inhibition curves were determined using six to nine different concentrations of adenine, spanning three orders of magnitude. Three separate experiments were performed, each in triplicate, unless otherwise noted. Nonspecific binding was determined in the presence of 100 µM unlabeled adenine. Incubations were carried out for 1 h at room temperature and terminated by rapid filtration through GF/B glass fiber filters (Whatman, Dassel, Germany). Filters were washed three times, 2 ml each, with freshly prepared ice-cold 50 mM Tris-HCl buffer, pH 7.4 and immediately transferred to mini vials. Scintillation cocktail (Ultima Gold, Canberra Packard, 2.5 ml) was added and after an incubation of 9 h filter-bound radioactivity was measured by liquid scintillation counting at an efficiency of 54%. In some experiments, the addition of mammalian protein was omitted and replaced by 100 µl of different buffer samples. For competition experiments with bacteria, either 100 µl of 1:100 or 1:1000 dilution of an overnight culture of the bacteria in Tris buffer, 50 mM, pH 7.4, or a membrane preparation of bacteria (containing 0.4–10 µg of protein) was used.

After isolation and classification of the bacteria from contaminated buffer solutions, experiments were performed with intact bacteria (approximately 6 × 10<sup>4</sup> bacteria/sample, which roughly equals 0.5 µg of total protein/sample), unless otherwise indicated, using the standard procedure (see above). The cell number was estimated from the optical density (OD) of the overnight culture suspension, and the assumption that OD<sub>600</sub> = 1 equals 3 × 10<sup>8</sup> cells/ml [16].

### Data analysis

Data were analyzed using Prism 4.03 (Graph Pad, San Diego, CA, USA). IC<sub>50</sub> values were determined by fitting data to sigmoidal concentration-inhibition curves. Results are presented as means ± standard error of the mean (SEM) from the number of observations.

### Isolation of microorganisms from contaminated buffer solutions and classification

Microorganisms were isolated from Tris-HCl buffer, pH 7.4, by plating 300 µl of the buffer on LB agar plates. Single colonies were isolated and incubated overnight in LB medium at 37°C with constant shaking at 230 rpm. Further separation was achieved by plating bacteria from overnight cultures on plate count (Merck, Darmstadt) and blood agar (Oxoid, Wesel). Morphology of colonies was visually examined. Conventional physiological and biochemical characterization assays, including gram staining, catalase and oxidase activity, motility, and oxidation/

fermentation (O/F) test were carried out and analyzed according to *Bergey's Manual of Systematic Bacteriology* using reagents from Merck (Darmstadt) [17]. In addition, the following kits and appliances were used: BBL OXI/FERM Tube II (Schwarz Pharma GmbH, Germany), API 20 NE strips and apiweb software (Biomérieux, Nürtingen, Germany), and VITEK 2 fully automated system (Biomérieux, Nürtingen, Germany).

## Results

### Protein dependence of [<sup>3</sup>H]adenine binding

As a first step, we reevaluated (unpublished) data obtained in initial studies that had been performed to investigate the suitability of [<sup>3</sup>H]adenine as a radioligand for labeling adenine receptors. Figure 1 shows [<sup>3</sup>H]adenine binding to different membrane preparations from (a) rat brain striatum, (b) rat brain cortex, and (c) HEK293 cells and CHO-K1 cells. For each membrane preparation, different amounts of protein were investigated (25, 50, 100, and 200 µg for striatum; 0, 25, 50, 100, and 200 µg for cortex; 50 and 100 µg for HEK and CHO cells). In all cases, we found a large, approximately linear, increase in specific binding with increasing protein concentration, whereas the increase in nonspecific binding was small. No specific binding was detected in the absence of protein (see Fig. 1b).

### Microbial contaminations in buffer solutions

As a next step, we investigated whether microbial contaminations present in incubation buffers that were not freshly prepared might be responsible for high counts occasionally observed in [<sup>3</sup>H]adenine binding studies in our laboratory. We performed a systematic analysis of buffer solutions (50 mM Tris-HCl, pH 7.4) stored at different conditions (different periods of time and temperatures), in different containers (plastic, glass, different sizes) used in our laboratory. Samples of buffer solutions were taken, and radioligand binding studies were performed with [<sup>3</sup>H]adenine (10 nM) using the same procedure as for the labeling of adenine receptors, except that no tissue or cell membrane preparation was added. Most investigated buffer solutions did not show any specific [<sup>3</sup>H]adenine binding. However, one sample of Tris buffer that had been taken from a 5-l plastic container with a small orifice, stored at 4°C, exhibited high affinity binding of [<sup>3</sup>H]adenine (data not shown). In contrast, radioligands used for the labeling of adenosine receptors ([<sup>3</sup>H]2-Chloro-N<sup>6</sup>-[<sup>3</sup>H]cyclopentyladenosine (CCPA) (A<sub>1</sub>) [18], [<sup>3</sup>H][<sup>3</sup>H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine (MSX)-2 (A<sub>2A</sub>) [19], [<sup>3</sup>H]8-Ethyl-4-methyl-2-phenyl-(8*R*)-4,5,7,8-

tetrahydro-1*H*-imidazo[2,1-*b*]purin-5-one (PSB)-11 (A<sub>3</sub>) [20]), or P2Y<sub>12</sub> receptors ([<sup>3</sup>H]2-propylthioadenosine-5'-adenylic acid (1,1-dichloro-1-phosphonomethyl-1-phosphonyl) anhydride (PSB)-0413) [21] did not show any specific binding to that buffer solution.

Figure 2 shows [<sup>3</sup>H]adenine binding determined in differently treated Tris-buffer solutions (in the absence of added protein). Freshly prepared buffer did not show any specific [<sup>3</sup>H]adenine binding. After storing the buffer for 1 day at 4°C, a small degree of [<sup>3</sup>H]adenine binding could be observed, and after 2 weeks, [<sup>3</sup>H]adenine binding was significant (562±19 cpm specific binding). There was a large, exponential increase in specific [<sup>3</sup>H]adenine binding with time, and after 6 weeks at 4°C, approximately 7,000 cpm (specific binding) were measured. After the 6-week-old buffer was filtered through 0.2 µm filters, [<sup>3</sup>H]adenine binding was completely abolished. Heating of the buffer for 1 min at 80°C dramatically reduced [<sup>3</sup>H]adenine binding, whereas heating for 3 min at 80°C or heating at 121°C for 20 min in an autoclave led to complete loss of specific [<sup>3</sup>H]adenine binding.

### Isolation of microbial buffer contaminants

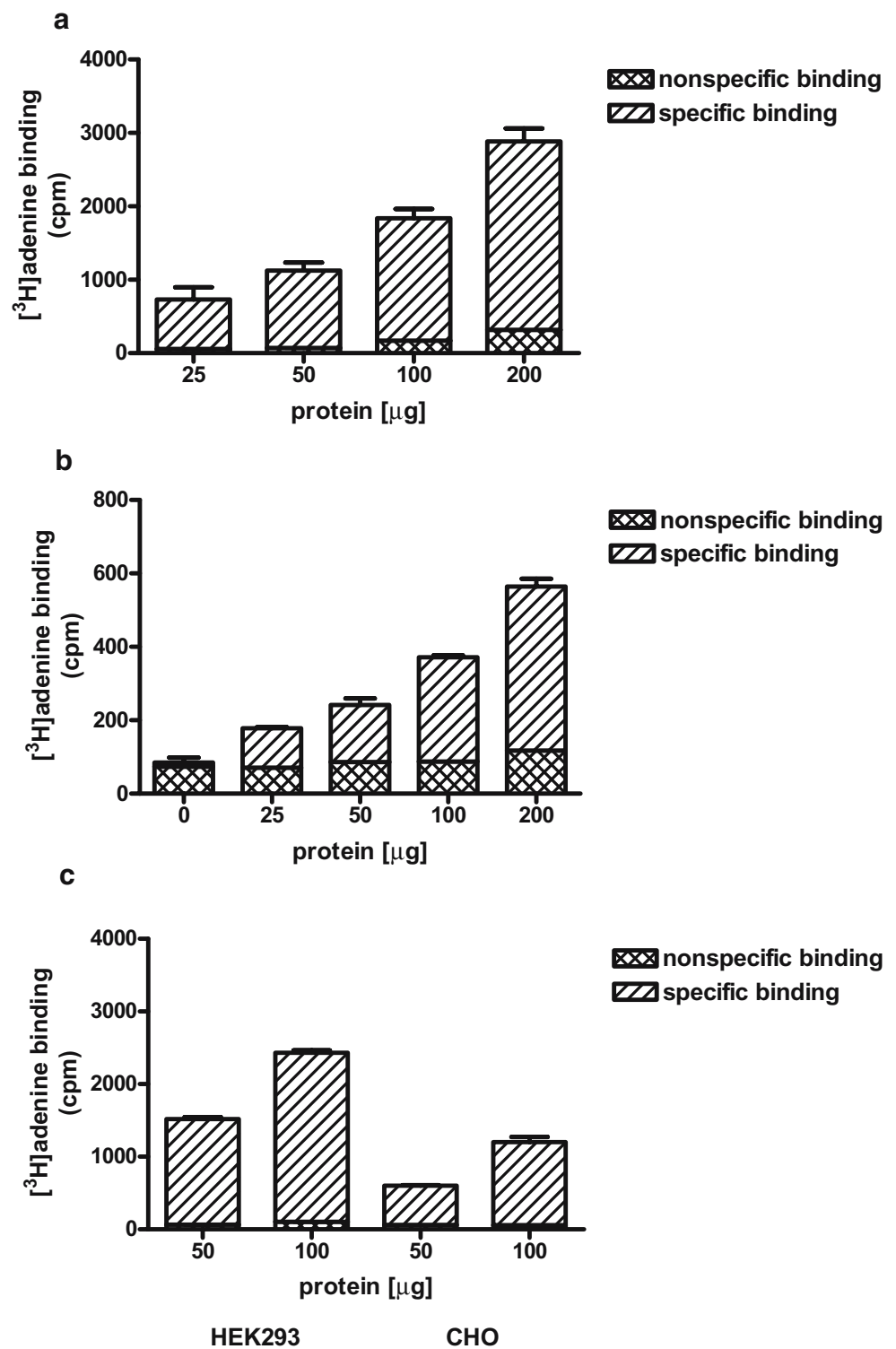
As our results indicated that adenine binding was due to microbial contaminations growing in the incubation buffer, we decided to isolate the contaminants in order to characterize and eventually identify them. The microorganisms were isolated by plating contaminated incubation buffer on agar plates. This led to identification of three bacterial strains differing in the morphology of the formed colonies. The three strains were then separately amplified in medium over night, and membrane preparations were obtained to perform homologous competition binding assays using [<sup>3</sup>H]adenine (Fig. 3a).

Membrane preparations of contaminant 1 showed a very high affinity for adenine (IC<sub>50</sub> = 4.59 ± 0.49 nM vs. 10 nM [<sup>3</sup>H]adenine, *n*=3), whereas membrane preparations of the other two contaminants appeared to have considerably lower affinities (contaminant 2: IC<sub>50</sub> = 1008 ± 240 nM, *n*=2; contaminant 3: IC<sub>50</sub>=2351 nM, *n*=1). For comparison, the binding curve for adenine at rat brain cortical adenine receptors (IC<sub>50</sub> = 57.0 ± 4.4 nM) is shown (Fig. 3a). Using amounts of membrane preparations of contaminant 1 that contained more than 1 µg protein/assay tube led to depletion of the radioligand (more than 50% of the added radioligand was bound to the protein) (data not shown).

### Identification of microbial contaminants

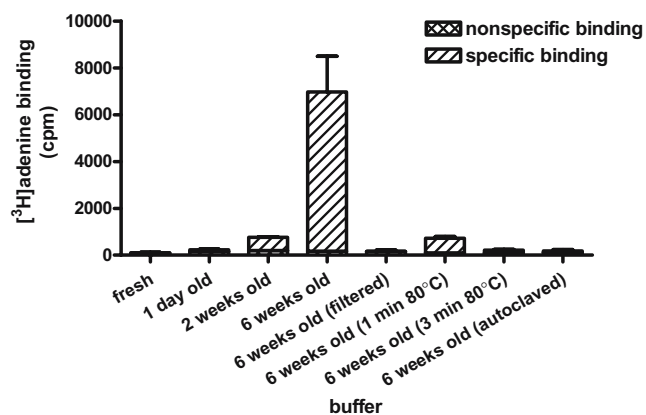
In order to identify the contaminants, standard procedures and classification kits were used (see Table 1). Standard tests, including catalase, oxidase, fermentation, motility

**Fig. 1** Protein dependence of [ $^3$ H]adenine binding to adenine receptors in rat brain striatal membranes (a), in rat brain cortical membranes (b), and in human embryonic kidney (HEK293) cell membranes and in Chinese hamster ovary cell (CHO) membranes (c). Different amounts of protein were incubated for 60 min with 10 nM of [ $^3$ H]adenine in Tris-HCl buffer, pH 7.4 ( $n=3$ ). Nonspecific binding was determined in the presence of 100  $\mu$ M adenine



test, and gram staining indicated that contaminant 1 is *Pseudomonas* spp. or a strain of *Achromobacter* spp., contaminant 2 is *Achromobacter* spp., and contaminant 3 *Acinetobacter* spp. [17]. All three bacteria are strictly aerobic gram-negative rods. For further classification, a standardized system (Api 20 NE) for classification of

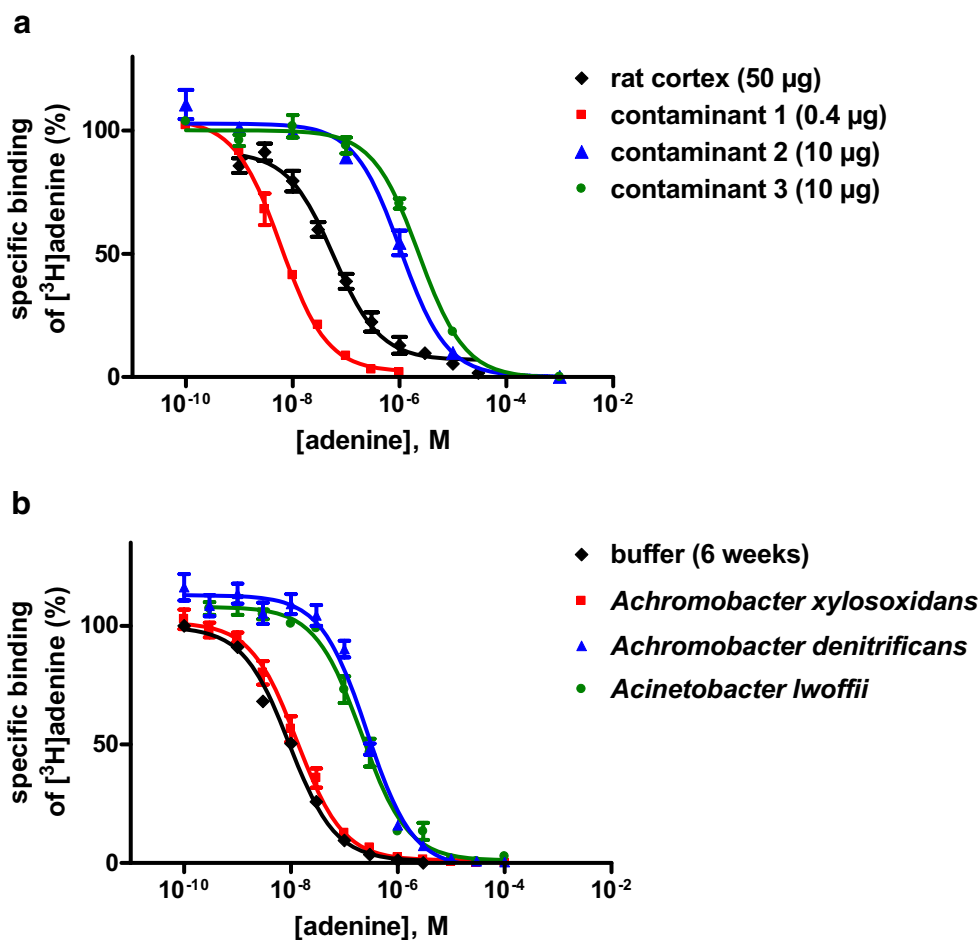
bacteria was used, which combines conventional and assimilation tests for identification of gram-negative rods not belonging to the Enterobacteriaceae. It was found that contaminant 1 was positive for oxidase, nitrate reduction, glucose degradation, gluconate, caprate, adipate, maltose, citrate, and phenylacetate, whereas contaminant 2 was



**Fig. 2** [ $^3\text{H}$ ]Adenine binding in the absence of added protein (cells or cell membranes). Incubation buffer (Tris-HCl, pH 7.4) was kept in a 5-l plastic container at 4°C for up to 6 weeks. Differently treated buffer samples (100  $\mu\text{l}$ ) were tested for [ $^3\text{H}$ ]adenine binding. The results shown represent means of three independent experiments  $\pm$  standard error of the mean

negative for glucose, and caprate and contaminant 3 only showed positive results for caprate, maltose, and phenylacetate. Those results confirmed contaminant 3, already presumed to belong to the species *Acinetobacter*, as *A. lwoffii*, with a probability of 98.1%, and led to

**Fig. 3** Competition curves for adenine versus 10 nM [ $^3\text{H}$ ]adenine obtained with membrane preparations from rat brain cortex and isolated microorganisms (contaminants 1–3) (a) and intact bacteria (b).  $\text{IC}_{50}$  values (a): rat cortex =  $57.0 \pm 4.4$  nM ( $n = 3$ ); contaminant 1 =  $4.59 \pm 0.49$  nM ( $n = 3$ ); contaminant 2 =  $1,010 \pm 240$  nM ( $n = 2$ ); contaminant 3 =  $2,350$  nM ( $n = 1$ ).  $\text{IC}_{50}$  values (b): *Achromobacter xylosoxidans* =  $13.8 \pm 2.7$  nM ( $n = 8$ ); *Achromobacter* =  $253 \pm 46$  nM ( $n = 5$ ); *Acinetobacter lwoffii* =  $299 \pm 129$  nM ( $n = 5$ )



the identification of contaminant 1 as *A. xylosoxidans* (94.5% probability) and of contaminant 2 as *A. denitrificans* (82.2% probability) (see Table 1). Identification of the two *Achromobacter* strains with the VITEK fully automated system for rapid bacterial identification and antibiotic susceptibility confirmed both as members of the *Achromobacter* spp. with a probability of 90% each; however, differences were found in the results for phosphate, citrate, and proline assimilation (for details, see Table 1). Table 1 summarizes selected test results, which were positive for at least one of the strains.

[ $^3\text{H}$ ]Adenine binding assays with isolated, intact bacteria

Adenine competition binding studies were performed using isolated, intact bacteria (Fig. 3b). The  $\text{IC}_{50}$  value obtained with whole bacterial cells of *A. xylosoxidans* ( $\text{IC}_{50} = 13.8 \pm 2.7$  nM) was in the same concentration range as that obtained with membrane preparations of the same bacteria, previously designated contaminant 1 ( $\text{IC}_{50} = 4.59 \pm 0.49$ ). For the other two bacteria, *A. denitrificans* and *A. lwoffii*,  $\text{IC}_{50}$  values were 5- to 10-fold lower when determined in whole bacterial cells compared with membrane prepara-

**Table 1** Summary of classification test results using standard procedures and kits

Test	Contaminant 1	Contaminant 2	Contaminant 3
<b>Morphology of colonies</b>			
Form	Round	Round	Round
Diameter	1–2 mm	1 mm	1 mm
Color	Grey	Beige	White
Brim	Smooth	Smooth	Smooth
Surface	Shiny	Shiny	Shiny
Cross section	Raised	Raised	Raised
Gram stain	Negative	Negative	Negative
<b>Biochemistry</b>			
Catalase	+	+	+
Oxidase	+	+	–
O/F test	–/–	–/–	–/–
Motility	+	+	–
Lysine/BBL Ox/Ferm Tube II	–	+	n.d.
Result	<i>Achromobacter</i> spp. or <i>Pseudomonas</i> spp.	<i>Achromobacter</i> spp.	<i>Acinetobacter</i> spp.
<b>Api 20 NE</b>			
NO <sub>3</sub> , nitrate reduction	+	+	–
GLU, glucose	+	–	–
GNT, gluconate	+	+	–
CAP, caprate	+	–	+
ADI, adipate	+	+	–
MLT, maltose	+	+	+
CIT, citrate	+	+	–
PAC, phenylacetate	+	+	+
OX, oxidase	+	+	–
Result	<i>A. xylosoxidans</i> (94.5%)	<i>A. denitrificans</i> (82.2%)	<i>A. lwoffii</i> (98.1%)
<b>VITEK 2</b>			
N-LGGH, $\gamma$ -L-glutamyl (nutrient assimilation, NA)	+	+	n.d.
P-BPHO, bis(p-nitrophenyl)phosphate	+	–	n.d.
C-CIT, citrate	–	+	n.d.
C-KGA, $\alpha$ -acetoglutaryc acid	+	+	n.d.
N-LPROT, L-proline-NA	+	–	n.d.
Result	<i>Achromobacter</i> spp. (90%)	<i>Achromobacter</i> spp. (90%)	n.d.

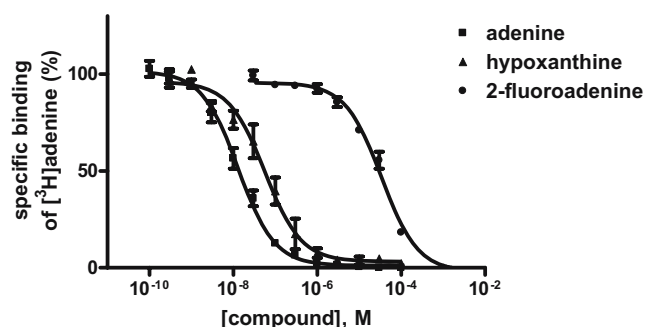
n.d. not determined

tions. From the homologous competition experiments,  $K_D$  and  $B_{max}$  values were estimated for *A. xylosoxidans* (membranes and intact cells). For bacterial membrane preparations, a  $K_D$  value of  $5.84 \pm 1.12$  nM and a  $B_{max}$  value of  $266 \pm 65$  pmol/mg protein was calculated ( $n=3$ ). For the intact bacteria, a  $K_D$  value of  $11.0 \pm 1.2$  nM and a  $B_{max}$  value of  $780,000 \pm 120,000$  sites/cell ( $n=8$ ) was obtained.

#### Structure–activity relationships

Affinities for selected compounds at adenine binding sites of *A. xylosoxidans* were determined in competition assays using whole bacterial cells and compared with data obtained in binding studies at the rat brain adenine receptor [7]. Figure 4 shows competition curves for selected compounds, which

exhibited high affinity, i.e., adenine ( $IC_{50} = 13.8 \pm 2.7$  nM), hypoxanthine ( $IC_{50} = 59.1 \pm 19.6$  nM), and 2-fluoroadenine ( $IC_{50} = 32,100 \pm 3000$  nM). The results for all compounds tested are summarized in Table 2. Whereas the affinity of adenine for the binding sites of *A. xylosoxidans* was in the same range as for the rat adenine receptor, the affinities for hypoxanthine and 2-fluoroadenine differed substantially from those determined for the rat adenine receptor. Hypoxanthine showed very low affinity for the rat adenine receptor ( $K_i$   $45,000 \pm 19,400$  nM), but high affinity for the bacterial [<sup>3</sup>H]adenine binding sites, with an  $IC_{50}$  value in the low nanomolar range ( $59.1 \pm 2.0$  nM). For 2-fluoroadenine, the opposite was true: the  $K_i$  value for rat brain cortical adenine receptor was  $620 \pm 140$  nM [7], whereas the  $IC_{50}$  value for the *A. xylosoxidans* binding site was in the micromolar range ( $32,100 \pm 3,000$  nM).



**Fig. 4** Competition curves for adenine, hypoxanthine and 2-fluoro-adenine versus 10 nM [<sup>3</sup>H]adenine obtained with *Achromobacter xylosoxidans* (intact bacteria) ( $IC_{50}$  values : adenine =  $13.8 \pm 2.7$  nM ( $n = 8$ ); hypo – xanthine =  $59.1 \pm 19.6$  nM ( $n = 3$ ); 2 – fluoro-adenine =  $32,11100 \pm 3000$  nM ( $n = 3$ ))

## Discussion

[<sup>3</sup>H]Adenine has been successfully used by us [6, 7] and other laboratories [5, 10] to label the recently discovered rat and mouse adenine receptors. However, problems with [<sup>3</sup>H]adenine binding assays have been reported by two laboratories [11, 12]. These have led to the suggestion that [<sup>3</sup>H]adenine was not a suitable radioligand for the labeling of G protein-coupled adenine receptors [11, 12]. IJzerman and coworkers [12] reported that [<sup>3</sup>H]adenine binding was not protein dependent and that it bound with nanomolar affinity to glass fiber filters in the absence of added protein. These results, which were contradictory to our own data, prompted us to reexamine the [<sup>3</sup>H]adenine binding results that we had obtained during the past years, trying to find an explanation for the discrepancies.

In contrast to the results reported by IJzerman and coworkers [12], binding of [<sup>3</sup>H]adenine to various membrane preparations was strictly protein dependent in our

hands, as expected (Fig. 1). Specific binding linearly increased with increased amounts of protein and thus increased numbers of adenine receptors. Nonspecific binding was generally low for [<sup>3</sup>H]adenine, and there was only a minor increase in nonspecific binding with increasing protein concentrations. In the absence of protein, no specific binding was observed (Fig. 1b). These results indicated that [<sup>3</sup>H]adenine was a suitable radioligand for labeling adenine receptors. In fact, we could recently perform [<sup>3</sup>H]adenine binding assays on a null background for the first time, namely, at the mouse adenine receptor heterologously expressed in Sf21 insect cells. Whereas membrane preparations of the nontransfected Sf21 cells did not exhibit any specific [<sup>3</sup>H]adenine binding, cell membranes prepared from cells infected with recombinant baculoviruses bound [<sup>3</sup>H]adenine with high affinity [6].

However, when we looked carefully at all of our previous [<sup>3</sup>H]adenine binding data, we found a few [<sup>3</sup>H]adenine binding experiments that could not be evaluated due to unusually high radioactivity counts. These occasional problems had been solved by carefully controlling the experimental conditions, e.g., by using freshly prepared buffer solutions. Stimulated by the experiences reported by the IJzerman group [12], we decided to perform a systematic study to find out the reasons for those problems, which might also be causative for erroneous [<sup>3</sup>H]adenine binding results in other laboratories [11, 12].

High affinity binding of [<sup>3</sup>H]adenine to filter paper, as suggested by IJzerman and coworkers [12], could be excluded by our experiments, as buffer solutions that were freshly prepared did not show any specific [<sup>3</sup>H]adenine binding in filtration assays using glass fiber GF/B filters, the same filters that had been used by the IJzerman group [12]. On the contrary, we discovered that bacterial

**Table 2** Comparison of affinities of adenine and selected compounds for the rat adenine receptor and bacterial binding sites determined in radioligand binding studies

Compound	<i>Achromobacter xylosoxidans</i> (intact bacteria)	Rat brain cortical membranes
	$IC_{50} \pm SEM$ [ $\mu$ M] <sup>a</sup> (% inhibition $\pm$ SEM at 10 $\mu$ M)	$K_i \pm SEM$ [ $\mu$ M] <sup>a</sup> (% inhibition $\pm$ SEM at 100 $\mu$ M)
Adenine	$0.0138 \pm 0.0027^b$	$0.0299 \pm 0.0034^c$
2-Fluoro-adenine	$32.1 \pm 3.0$	$0.62 \pm 0.14^c$
2-Hydroxyadenine	$\gg 10$ ( $7 \pm 15$ ) <sup>d</sup>	$29.4 \pm 6.4$
2,6-Diaminopurine	$\gg 10$ ( $3 \pm 8$ ) <sup>d</sup>	$4.95 \pm 0.75$
1,N <sup>6</sup> -Ethenoadenine	ca. 10 ( $53 \pm 6$ ) <sup>d</sup>	$34.4 \pm 8.1$
Hypoxanthine	$0.0591 \pm 0.0196$	$45.0 \pm 19.4$
Uracil	$\gg 10$ ( $6 \pm 1$ )	$\gg 100$ ( $-6 \pm 2$ )
Xanthine	$> 10$ ( $17 \pm 6$ )	$> 100$ ( $23 \pm 7$ )
Uridine	$\gg 10$ ( $5 \pm 5$ ) <sup>d</sup>	$\gg 100$ ( $-9 \pm 3$ )
Adenosine	$\gg 10$ ( $12 \pm 9$ ) <sup>d</sup>	$19.4 \pm 6.3^c$

<sup>a</sup> Results are from three independent experiments performed in triplicate, unless otherwise noted

<sup>b</sup> Results are from eight independent experiments performed in triplicate

<sup>c</sup>  $K_i$  values and % inhibition values are from [7]

<sup>d</sup> Results from two independent experiments performed in triplicate



contaminations, which can be present in buffer solutions, express high-affinity binding sites for [ $^3$ H]adenine and therefore impede adenine receptor binding assays. When we examined different buffer solutions stored in our laboratory, we discovered high [ $^3$ H]adenine binding in Tris-HCl buffer solution (pH 7.4) stored at 4°C in a 5-l plastic container. That container had only a small orifice and was therefore difficult to purify. Thus, microbial contamination in this container was carried over when new buffer solution was prepared. When the contaminated buffer was filtered through a bacteria-tight filter (0.2  $\mu$ m) or heated in order to denature proteins, specific [ $^3$ H]adenine binding was abolished, strongly indicating that microbial proteins were responsible for the high affinity binding of adenine. A further indication that a living organism was involved was the fact that adenine binding increased exponentially with time. Interestingly, the microorganisms grew better at 4°C than at room temperature.

Our first presumption, that the contaminants might belong to yeast, could not be proven. *Saccharomyces cerevisiae* was used as a control organism for binding studies but showed no [ $^3$ H]adenine binding (data not shown). Three microorganisms were isolated from the contaminated Tris-buffer solution and identified using standard procedures and kits. Two of these contaminants were assigned to the genera *Achromobacter* and one to *Acinetobacter*. Both bacteria species are gram-negative rods and are strictly aerobic. They are commonly found in soil and water [17]. For healthy humans or animals, they are not pathogenic, but especially *A. xylosoxidans* and *A. lwoffii* have gained increasing importance due to their ability to cause nosocomial infections [22–32]. These bacteria are able to grow under nonoptimal conditions, such as low temperature and restricted nutrient supply [17, 24–26]. They are able to metabolize a wide variety of organic substances, such as chemical pollutants in the environment, and can therefore be used as bioreporters and for the degradation of pollutants [33–36]. This is consistent with the fact that these bacteria are able to grow in simple Tris-HCl buffer at low temperature.

Both *A. lwoffii* and *Achromobacter* spp., exhibit specific binding sites for adenine, with IC<sub>50</sub> values as low as 13 nM for *A. xylosoxidans*. Thus, the detected adenine binding site in *A. xylosoxidans* exhibits an even higher affinity than the rat (29.9 nM [7], 18 nM [5]) or mouse (54.9 nM [6]) adenine receptor. The binding affinities for *A. lwoffii* and *A. denitrificans* were more than 70-fold lower, with affinities in the low micromolar range (1  $\mu$ M and 2.4  $\mu$ M, respectively) when membrane preparations were analyzed and about 20-fold lower when intact cells were investigated for binding (299 nM and 253 nM, respectively). For *A. xylosoxidans*, only a threefold difference was found when binding affinities for membranes were compared with those with intact cells (Fig. 3). The specific, high-affinity

[ $^3$ H]adenine binding site in *A. xylosoxidans* appeared to be expressed in extraordinarily high density, as amounts of membrane preparations that contained more than 1  $\mu$ g of protein/assay tube led to depletion of the radioligand (i.e., more than 50% of the added radioligand was bound to the protein). Rough estimations of receptor densities by homologous competition assays confirmed the high expression levels.

So far, the nature of these high-affinity adenine binding sites in bacteria is not known. However, bacteria express a large number of transporters, including nucleobase transporters, in order to secure their nutrient supply (for review see [37]). Nucleobase transport in bacteria has been mainly studied in *Escherichia coli* and *Bacillus* spp., as well as in the fungi *Aspergillus nidulans* and *Neurospora crassa* [37–39]. Usually, these transporters fulfill two main functions. Firstly, purines can serve as preformed bases for nucleotide biosynthesis, and secondly, they serve as nitrogen sources [40, 41]. Distinct adenine uptake systems have been identified, e.g., in *E. coli* [42]. The high density of the detected [ $^3$ H]adenine binding sites in *A. xylosoxidans* would be consistent with its function as a purine transporter and points to an important role of this protein, which appears to be upregulated when the bacteria are transferred to Tris buffer (unpublished observation), a medium poor in nutrients. Such an effect has been described for protozoa [43]: purine salvage enzymes and transporters can be dramatically up- or down-regulated according to growth stage and availability of purine sources [44]. Examples are the high-affinity hypoxanthine transporter in *Trypanosoma brucei brucei*, which shows a 450% increased transport rate after 24 h of purine deprivation, and the adenine transporter in *Crithidia luciliae*, which shows a >100-fold increase of adenine uptake after purine starvation [44, 45].

For *E. coli* as well as for *B. subtilis*, two adenine transport systems have been described: a low- and a high-affinity transport system [42, 46, 47]. The latter system is important when the concentration of adenine is low [46, 47]. Differences in adenine-binding affinity observed in our studies when intact cells were compared with membrane preparations (Fig. 3) might be explained by the existence of different transporters in the bacteria (*Achromobacter* and *Acinetobacter*). Whereas bacterial membrane preparations were obtained directly from an overnight culture grown in complete medium, binding studies at whole bacteria were performed after growing them in Tris-HCl buffer, a nutrient-poor medium, in which they may have upregulated the high-affinity transporters [42, 47].

In order to investigate the structure–activity relationships of the high-affinity adenine binding site in *A. xylosoxidans*, a series of compounds, including adenine derivatives, other purines, and pyrimidines (uracil, xanthine, hypoxanthine), and nucleosides (uridine, adenosine) were investigated in binding studies, and the results were compared with those

obtained at the rat adenine receptor. As expected, structure–activity relationships at the rat adenine receptor were very different from those at the bacterial adenine binding site. Hypoxanthine, for example, which exhibits a low affinity for the rat adenine receptor (45,000 nM), bound to the bacterial site with 760-fold higher affinity (59.1 nM), and 2-fluoroadenine, which showed a high affinity for murine adenine receptors (620 nM), bound with a 50-fold lower affinity (32,100 nM) to the bacterial site. Several compounds that had shown affinity in the micromolar range at rat adenine receptors (adenosine, 2-hydroxyadenine, 2,6-diaminopurine) were completely inactive at *Achromobacter* binding sites.

Alexander had previously reported that hypoxanthine at a concentration of 10  $\mu$ M completely blocked [<sup>3</sup>H]adenine binding to rat brain membranes in his experiments, the results of which were not consistent with the labeling of a G protein-coupled adenine receptor [11]. It might be speculated that he actually labeled a bacterial adenine binding site rather than the rat adenine receptor, which would explain the discrepant results. The limited number of compounds (five) investigated by IJzerman and coworkers [12] do not allow a full comparison of the structure–activity relationships, but large differences were observed for two compounds—5'-deoxyadenosine (725,000 nM [12] vs. 823 nM (rat adenine receptor) [10]) and 2-fluoroadenine (19,000 nM [12], 620 nM (rat adenine receptor) [7])—indicating the labeling of a very different, presumably a bacterial, binding site by the authors [12]. The fact that hypoxanthine exhibits high affinity for the [<sup>3</sup>H]adenine binding site in *A. xylosoxidans* in the same concentration range as adenine itself is another indication that the labeled protein may be a purine transporter.

Nucleobase transporters have been identified in bacteria, fungi, protozoa, algae, plants, and mammals, but only few have been cloned and analyzed in detail [37, 48]. Five basic families of nucleobase transporters have been described: the nucleobase-ascorbate transporters (NAT), which include members from archaea, eubacteria, fungi, plants, and metazoa; the purine-related transporters (PRP), which are restricted to procaryotes and fungi; the purine permeases (PUP), which are purine transporters exclusively found in plants; and the equilibrative (ENT) and concentrative (CNT) nucleoside transporters, which not only transport nucleosides but may also transport nucleobases [37, 48–52]. Bacteria have developed different transport systems for related compounds, which allow them to independently absorb those compounds. This is an advantage when growing under nutritional deprivation [46, 53]. For *C. luciliae*, a nucleobase transporter that recognizes adenine and hypoxanthine equally well has been described [45]. In *E. coli*, adenine and uracil have different, specific transport systems, as do xanthine and guanine, whereas hypoxanthine might

utilize the guanine transporter [53]. For *B. subtilis*, specific transport systems for guanine and hypoxanthine, for guanosine and inosine, as well as three independent uptake systems for adenine, adenosine and uracil, have been identified [46].

From the described observations, we conclude that the high-affinity binding sites found in bacteria isolated from Tris-buffer solutions have not much in common with the adenine receptors found in mammals. The genomes of the bacteria *A. baumannii* and of several closely related bacteria, such as *Pseudomonas* spp. are known. Therefore, we performed a search to identify potential sequences with homology to the rat and mouse adenine receptors, which, however, yielded no hits. It appears likely that [<sup>3</sup>H]adenine labels a high-affinity nucleobase transporter for adenine and hypoxanthine in *Achromobacter* spp., a bacterium isolated as a contaminant from Tris-HCl buffer. The high affinity in the low nanomolar range is in fact unusual; therefore, it may be speculated that the labeled protein might belong to a new type of high-affinity bacterial nucleobase transporter.

## Conclusions

In conclusion, we have demonstrated that [<sup>3</sup>H]adenine is a well-suited radioligand for the labeling of G protein-coupled adenine receptors, but precaution is advised for preparing and storing buffers used for the assays to avoid bacterial contaminations. After systematically analyzing occasionally encountered irregularities in [<sup>3</sup>H]adenine binding assays, we were able to isolate three bacteria, commonly found in soil and water, from Tris-HCl buffer. They were identified as *A. lwoffii*, *A. xylosoxidans*, and *A. denitrificans* and revealed high-affinity binding sites for [<sup>3</sup>H]adenine.

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